

Truth at any price

GEORGE STEINER, in his recent televised Bronowski Memorial Lecture, asked "Has truth a future?" For he fears that there may be a fundamental incompatibility between man's hopes of justice and decency and certain categories of truth.

Steiner, whose lecture is published by the BBC at £1, remarks that an infatuation with truth-for-its-own-sake emerged in Greece and Greek colonies between the seventh and fifth centuries BC, and must be clearly contrasted with the pursuit of useful knowledge—an occupation of many times and many places. "The man prepared to risk destitution or ridicule in order to solve a quadratic equation or a paradox in logic is an Ionian or south Italian Greek in the age of Pythagoras and Anaximander, of Heraclitus and Anaxagoras. Those who today pursue abstract truths in the sciences, in mathematics, in philosophy, are heirs to his hunger."

But, continues Steiner, abstract thought and the pursuit of truth have had their detractors, even enemies. Mystics and irrationalists, dogmatists and supernatural revelationists all in their different ways see mathematics, science and other scholarly pursuits as of a lower order than knowledge revealed through ecstasy or a divine statement. Then there are the romantic existentialists, Blake, Wordsworth, Kierkegaard, Tolstoy and so on, for whom 'the values of ecstatic self-realisation, of moral goodness, of simplicity, even of absurdity are set high above those of mathematical-scientific truth'. Finally, there are the truth-is-not-neutral critics who assert that truth and logic is used as a class weapon of oppression. There can be found mathematicians and scientists, Steiner might have added, holding with unmistakable sincerity all of the above views.

Now, however, we are confronting a new challenge of a rather different kind, he claims. Some of the truths that we turn up or might be capable of turning up could be in conflict with ideals of social justice or even survival. We know, as an extreme example, that sometime in the remote future the universe will run down in some way or other, and that will be the end of us. This piece of information is tolerable to most people and does not lead to any great move to restrict cosmologists' freedom of action. But some things are much more immediate. Predetermination of the sex of the foetus, cloning, the contributions of heredity and environment—such matters are the stuff of present-day research and the results of this research could have immediate implications for the social system we know. Many would argue that we are within sight of revealing verities of a kind that society cannot handle, and that now is the time for a socially responsive science, scaled down to human needs. "Not the costly elitist chase after the esoteric and possibly destructive fact, but the arcadian pilgrimage towards self and community."

But it won't work, claims Steiner; partly because nation-states have now come to believe that future grandeur and security might depend on unrestricted investigation, but mainly because "the obsession with objective and abstract truth is imprinted on the western mind", and even if truth lies in wait for us with hideous snares, there will always be those who will press on. Bronowski, humane and learned

man, might have said that "if man's endurance as a more of less decent creature depends on leaving certain doors closed, then so be it", thinks Steiner. But the other point of view, that truth matters more than man, is "also possible".

This is not unfamiliar territory; for instance last August Sir Andrew Huxley was asking similar if more specific questions about research into the heritability of IQ, and nuclear physicists have agonised over morality and research for more than thirty years. But it can rarely have been raised in a more penetrating and cross-cultural way. Yet for all that, it is possible that however carefully balanced and scrupulously worded Steiner's argument is and however agonisingly he has had to choose to come down on the side of truth, even against man, the picture that he has painted of the emotive concept 'truth' is altogether too stark, and the choice between man and truth made much too clear-cut. If one were to ask most scientists how they view their profession, they might well say something about hoping to add to knowledge, or conceivably might talk about making falsifiable hypotheses or learning by trial and error. But the word 'truth' might never crop up. Partly this would be because of the debasement of it by the propaganda and publicity world. But more likely it would be because the spirit of scientific enquiry tends not to reveal truths so much as to answer some questions at the expense of raising others of a more fundamental character. For sure a question such as 'is it true that the sex of a child can be pre-determined?' may well be answerable in the affirmative in the foreseeable future, but such a question is more one of technique not an eternal verity. Steiner persistently conjoins mathematics with science in his lecture, indeed uses mathematics for the examples of the elucidation of the earliest truths, and this may well be a mistake. The rigorous logical consequences of mathematical enquiry have very little in common structurally with the results of biological enquiry.

Nor is this the only ambiguity that comes from too stark a portrayal of truth. Those scientist who think seriously about marking certain doors 'too dangerous to open' are by no means all committed to "putting the questing, autistic brain to pasture, while instinct plays" or even to an "alternative style of cerebration" as Steiner would have it. They still do recognisable research, sometimes highly esoteric; they still submit papers to journals, live with peer review and hope to outpace their rivals—in short they behave like other scientists, and if truth were the right word for it, they would equally be reckoned to be pursuing truth, but not necessarily truth at any price.

Many years ago scientists recognised some sorts of experiments with humans and animals as ethically unacceptable. The doing of such experiments might yield all sorts of valuable knowledge but at an uncertain expense of suffering or degradation. So 'truth' was retarded, but biomedical researchers are not the less dedicated to the pursuit of knowledge for almost universally honouring this ethical code. We may at present be groping towards a similar code for the protection of society, and just possibly in a hundred years time our successors may deem the world of learning to have been little hurt by it. □



Saudi Arabia and the travelling scientist

ONLY in the past week has the outside world come to hear of the particularly savage public executions in Saudi Arabia last November of a princess and her husband for, apparently, contracting a marriage contrary to royal decree. At least the couple were spared being stoned to death—a fate which befell three men in Saudi Arabia last year after they had been found guilty of sexual offences. The Islamic code of justice has harsh things in store for sexual transgressors, and Saudi Arabia is not, of course, the only country that operates such a code.

But why is this any special concern of the readers of *Nature*? Certainly we should be exercised when states interfere with the elementary human rights of scientists or when they tamper with the means of scholarly communication. But is this recent matter, however barbarous and seemingly unjust by our different moral and legal standards, any cause for scientists to take special interest in?

The answer is yes. Saudi Arabia is in the throes of a mighty transition, often described as a leap from the Middle Ages to the atomic era. Science, technology and medicine are playing an enormous role in effecting this change, and higher education—non-existent before 1947—is now open to several thousand students each year. What is more, Saudi Arabian interests are not exclusively in applied science;

there has been talk of a particle accelerator and rather more than talk about a major telescope. All of this has meant an increasing flow of Western scientists into the country to teach, collaborate and advise.

Such scientists have an easy and relaxed access to the Saudi intellectual community—easier, probably, than that of any other group, as they are not there specifically to sell equipment, buy oil or represent the delicately balanced interests of their home country. They thus have an excellent opportunity for quiet and honest conversations with hosts about the way that Saudi Arabia looks from the outside world and how decades of impressive achievements can count for little if there are not comparable developments towards at least the elements of a society that is humane and just.

Much informed opinion in Saudi Arabia would probably already concur with all this, but its hand would be immeasurably strengthened in pressing for change were the message to be repeated from the outside as often as possible. And there are several other countries where, equally, the travelling scientist can be a power for good in this way. Maybe we underestimate the influence that the global scientific community could have with its liberal attitudes and ease of access to people of influence. □

Medical education and biomedical research: uneasy equilibrium

George A. Silver, Professor of Public Medicine in the School of Medicine, Yale University, argues that medical education should be separated from research if the best doctors and research workers are to be produced.

AMONG the casualties of the upheavals of the 1960s, one of the most far-reaching has been the dissipation of unlimited faith in the benevolence of science and the efficacy of progress along with a loss of confidence in social institutions generally. Biomedical research and medical education have both suffered the consequences, although in different ways and to a different extent. There is dissatisfaction with the medical care system, a dawning recognition that not all diseases may be curable and scepticism of the motives of both medical educators and researchers.

A medical school dean writes (Sherman M. Mellinkoff *Johns Hopkins Med. Jour.* 141, 167; 1977): "Triumphs of the biomedical sciences have burgeoned exponentially. Nevertheless there sometimes seems to be a miasma of something between apprehension and gloom over the American medical schools of this decade. Seldom has medical education been subjected to so much criticism, ranging from valid to vicious, from thoughtless to mindless. It is as though success had earned no laurels but a handful of nettles." And he attaches a litany of the public and popular complaints, among which the outstanding are "... that medical students are deliberately selected or molded to be overly scientific and lacking in altruism" and that the schools "... pay too much attention to abstruse problems and neglect common ailments ..." both of which he considers false.

In searching for the culprit, if there is any, the questioning seems to centre on this intertwined relationship, the apparently indissoluble bond, between biomedical research and medical education. A measure of obloquy has in recent years fallen on Abraham Flexner, the educator whose report on medical education in the United States (*Medical Education in the United States and Canada*, Carnegie Foundation for the Advancement of Teaching, NY (1910)) is considered the platform from which modern medical education was launched. Although Flexner epitomised a tide then running and so gave his name to the course of events already in train, he should only bear responsibility for recognising the trend and helping establish a rational base for medical practice. He did propose the university as the locus for medical education and medical research as an important associated activity.

But 'research' in the early years of this century was not the research we know today. One must visualise Sir William Osler, observing patiently and critically, recording the natural history, symptomatology and autopsy evidence of disease as the prototype of the investigator of that day. There was only modest and inexpensive equipment in the laboratory for examination and study. In short, 'research' was essentially clinical study in a scientific style.

Now that we have a multimillion-multibillion dollar research industry fastened upon the medical education process throughout the academic world, we begin to have second thoughts. Examination of the medical research and medical education budgets for 1975, shows that while \$800 million went specifically for research, only \$368 million went into teaching and training in the academic institutions. Total budgets for the medical schools were twice this amount, including many other kinds of payments, but

research costs are heavy elements.

There has never been any secret about the fact that the goals of the research community have been to use the medical educational establishment to further its goals. "The simply stated goal of the NIH has always been to develop a science base for the formulation and solution of medical problems" (James A. Shannon *Jour. Med. Educ.* **51**; 1976).

For the proponents of the doctrine of the union of research and medical education, the course is clear. "If the definition of the goal of medicine as the saving of life, the relief of pain and the prevention of disability is accepted, it then follows that the tight coupling of biomedical science and clinical medicine is the critical feature of the medical school" (Donald W. Seldin *Clin. Res.* **23**, 280; 1975). And dilution of this bond is reprehensible: "[the introduction of community services] has had the effect of changing medical schools more and more into second rate public utilities . . . compromised as institutions for the education of students in the biomedical sciences." If biomedical research distorts medical education and medical education is a constraint on biomedical research, should the two be bound together? The international implications are far-reaching, in view of the world-wide imitation of the American design.

There are other voices. In a recent conference, some influential medical educational leaders expressed concern: "There are many signs that society is finding the medical profession too insensitive to health care", stated one medical school dean. Students seem to be selected, educated, engineered toward goals of scientific excellence that are only remotely related to the everyday needs of citizens. The participants were told that only 35% of faculty time was spent in basic instructional activities and they wondered if more time couldn't be devoted to teaching in order to reduce the phenomenal faculty to student ratio in this country. Ronald Christie, a dean, professor and clinical practitioner on three continents in a review of medical education world wide (*Medical Education and the State* DHEW publ. no. (NIH) 76-943 (1976)) expressed some doubts about the almost one-to-one faculty student ratio in the US, also pointing out that in Canada the ratio is one-to-three; in Australia one-to-ten and in the UK one-to-sixteen. Since these countries seem to graduate excellent doctors, on a par with the US product, the discrepancy—and the expense—is puzzling. The cost of medical education, per student per year averaging \$12,650 in the US when studied by the Institute of Medicine of the National Academy of Sciences, and ranging quite widely, from \$6,900 to \$18,650 (*Cost of Education in the Health Professions* IOM (NAS) DHEW publ. no. (HRA) 74-32 (1974)) seems to be twice as high as Canada's per student cost; three times Australia's and five times that of the UK.

If cost alone were the factor, one might hardly quarrel with the result, since the US is notoriously lavish in its expenditures. But the Christie note on the value of research as a concomitant of medical education, with which he heartily concurs, also queries the calibre of the research that is too often done to satisfy the requisite standard without providing the product required. He calls the mandarins to order in his comment that "Few of those involved in research in academic departments will admit even to themselves that their research is pedestrian or unprofitable . . ."

And a university president states (David S. Saxon *Jour. Med. Educ.* **51**, 991; 1976) in connection with the shibboleth of academic tie as the *sine qua non* for medical education: ". . . these opportunities (for mutual exchange of ideas and research advances between the medical and general campuses) are rarely exploited."

A science administrator points out (William D. Carey *Science* **197**, 825; 1977) how the tight bond between the academic, medical, educational and research areas may be

damaging to research activity: ". . . built-in inefficiencies and distractions are sapping the vitality of the research process . . . the dollars allocated to basic science no longer tell us much about the true levels of the research effort."

More grave a charge is levelled by a social scientist studying medical ethics in the practice of biomedical research (Bernard Barber *et al. Research on Human Subjects*. N. Y. Russell Sage; 1973). He quotes Merton, "The culture of science is, in this measure, pathogenic. It can lead scientists to develop extreme concern with recognition . . ." And he himself adds, ". . . we have presented evidence that the pressures of having to establish oneself in a competitive scientific community seem to have the effect of making these researchers engaged . . . less sensitive . . ." And again, ". . . our findings make it clear that it has some negative consequences for other important human values . . ."

If this is the attitude encouraged among researchers, should it prevail among students of medicine? Today's medical education makes the doctors much more dependent upon the technology of medicine, not only serving to reduce contact with and interest in the patient as a person, but also generating enormous expense in medical practice. Reinhardt ('Health Manpower Policy in the United States'. Paper presented at the University of Pennsylvania Bicentennial Conference on Health Policy, 11-12 November, 1976. Quoted in Somers and Somers, 'A Proposed Framework for Health Care Policies'. *Inquiry* **14**, 115-170, June 1977) estimates that each physician in the US is the source of \$260,000 of medical expenditure each year.

Few rational people in this century would want to dispense with important, tested and proven advances in medical science and practice; nor would they propose discontinuing the admirable research efforts of this and other countries. The question at issue is, need biomedical research be so intimately, inextricably bound to medical education? It is not irrational to ask whether the medical educational process could not be carried on separate from, though with useful liaison to, the biomedical research efforts. The changed emphasis would allow a different selection, admission and process, less emphasis on technology and more emphasis on the patient as a person. It might help to redirect physicians in their practice, reduce the volume and cost of medical care, without increasing the mortality or disability rates among Americans. To be sure, some are already questioning the efficacy of medical care in reducing morbidity and mortality in any case (John B. McKinlay and Sonja M. McKinlay *MMFQ/Health and Society*; 1977).

On the research side, the separation might be beneficial as well. Investigators, choosing a career in which they could advance without competing for academic title, would not be split between the burdens of teaching, for which they never have enough time, and the necessities of the research activity, for which more constant and undivided attention is also demanded. Less money would be wasted in routine 'research' whose sole purpose is justifying the medical educational/research mix. As the students' role-models change, medical-practice careers will change. As Barber says (*NEJM* **295**, 939; 1976), "Medical students are bright and ambitious; they have no difficulty ascertaining which subjects are taken seriously by the prestigious senior members of the faculty".

Biomedical research and medical education need to be separated to save them both from becoming isolated from the realities of the social setting, condemned to declining support in a climate of hostility in what should be their natural constituency. There is need for special attention to both. A medical education that glorifies research while ostensibly preparing students for practice will provide society with neither the best doctors nor the best investigators. □

Greek orthodoxy

The first of two articles commenting on the research policy and universities of Greece by **E M Pantelouris** describes the patriarchal and bureaucratic structure of Greece's higher education system.

WHY, one may ask, do 30,000 Greek students, representing nearly 30% of the student community of Greece, study abroad? Is this a vote against Greek higher education? Or are there just too few places for too many students? To describe the system is an answer in itself, and I shall attempt to do that here.

The aspiring Greek student cannot rely on the education provided by his or her six years of secondary schooling to secure a university place. Entrance is by written examination, conducted uniformly throughout the country, and to prepare effectively for these examinations, secondary school pupils commonly attend classes run by private cramming schools, which are a growth industry in Greece. Quite often, a young man or girl will spend a whole year after coming out of secondary school attending these 'frontisteria' before attempting, or re-attempting, the university entrance examination. Pressure is high, as the 1977 figures illustrate: slightly over 13,000 places were available for some 78,000 applicants (see Table 1).

This first hurdle over, the successful candidate embarks on a degree course that lasts as a rule four years (more for medicine and for some branches of engineering). Degrees are rather general, with six or more subjects continued to the fourth year. Lectures are formal and *ex-cathedra*, a format aggravated by the relative absence of a large body of published books in science subjects, and the extremely poor library facilities. Textbooks prepared by the professor in charge of each course are produced and distributed free by the government. This is very helpful, as few textbooks can be published economically in Greek, but might fossilise lecture courses.

There is nothing comparable to the British 10:1 student-staff ratio. No average figure can be given, but however calculated, the ratio would be several times higher. The professor of a 'chair' is the only one officially entitled to lecture to students, except for one or two 'authorised' sub-professors (*ifigitis*). The rest of the staff are kept incommunicado, except for the laboratory sessions. It has been put to me that they are selected as demonstrators rather than lecturers: most of them still have no higher qualification than a primary degree. Teaching is thus concentrated in the hands of the professor.

Dr E. M. Pantelouris visited Greece as a Nature travelling fellow.

Table 1 Entrants by examination to university level institutions†, 1977-78

Law*	2,896	] total 6,036
Economics*	3,140	
Literature, philosophy	1,145	] total 2,030
Theology	100	
Modern Languages	785	
Scientific and agricultural		4,329
Medicine, pharmacy, veterinary science		1,017
		total 13,412

*About a third of law and economics students are gainfully employed and hence in effect part-timers. For comparison, in the UK about 30% (212,000 including 49,000 of the Open University) of students—including postgraduates—are part-time

†Excluding physical education, teacher training and military schools

Table 2 An estimate of the number of Greek students abroad (before 1976)

Student population in Greece at university level	80,000
Total of Greek students abroad*	30,000
Annual student entry at universities abroad	6,000
Distribution of Greek students abroad:	
Italy	55%
UK	14%
France	8%
Germany	6%
USA	6%
Others	11%

*Based on applications in 1975 for foreign exchange for the purpose of financing full-time studies abroad

Academics and bureaucracy

Academic staff are also utterly dependent on the professor for tenure. Appointment to an assistantship lapses after nine years, unless a doctorate is obtained—an invitation to professors to grant internal doctorates to some to save them from redundancy. After a further six years, it becomes necessary to submit another thesis for the title of *hyphigitis* (or 'dozent'), some of whom may eventually be entrusted with a course or part of a course. Laboratory facilities, and acceptance of theses for such in-service internal degrees, depend in practice on the discretion of the professor, and reflect more his decisions about continuation of employment rather than any independent assessment of research ability.

Unavoidably this archaic system lowers the level of undergraduate teaching and prevents intellectual communication between staff and student. The students' response is disenchantment with the system, and the short term attitude that one is there just to get a degree of sorts, as nothing else

can be got—unless it is the intensive politicisation reflected in the slogans that completely deface the walls of university buildings.

Of course, students and staff are aware of the shortcomings of the system, care about it, and agitate. And it must be stressed that this system is breached by commonsense in many departments, where younger professors begin to bring to bear the approach under which they themselves trained abroad in the Anglo-Saxon world or elsewhere. But there remains in all cases the stranglehold of the greatest scourge of Greece: bureaucracy. If a professor wishes to authorise his second-in-command to give a course of lectures, the move has to be approved by somebody 'at the ministry', who will first calculate whether the lecturing load of the professor entitles him to such help—because an honorarium will be paid. Similarly, after an annual grant has been allocated, the department still has to submit for approval a list of purchases before they can be effected; so that work can be delayed for months because a chemical cannot be purchased outright. I have come across cases where the professor pays from his pocket to save the situation.

Here we come to the real key obstacle to reform: the tradition by which university regulations are nearly uniform for all institutions, and acquire their force through the ministry and publication in the government *Gazette*; that is through a centralised extra-university and political organ. University independence is restricted; and in abnormal periods—of which fortunately the present is not one—professors are dismissed or appointed by the will of the minister.

Yet paradoxically, the key to improvement lies with the government. If matters were left to the ability of universities to renew themselves, the impression is that the older Athens institutions would remain static, although the outcome could be different at the newer University of Thessaloniki. In one institution with some 25 professors I was told that only a handful favour reforms. To understand these attitudes it has to be taken into account that the most influential groups of professors are those in fields such as medicine and engineering, where titles of professor, *hyphigitis*, and so on are springboards for lucrative private practices, consultancies, and membership of innumerable committees.

A law is promised to replace the 'chair' system with a so-called sector system. Students and staff understand this to mean independence of university teachers in matters of lecturing and marking in their speciality, choosing their own research and participating in the running of academic affairs. The long incubation of the law, however, is giving rise to suspicions that the sectors will emerge as nothing more than groupings of related chairs as before.

It is to be hoped that the government may in the end come down on the side of actual, not simply cosmetic, changes that might enable Greek universities to gradually become something beyond mass producers of graduates, and rise to be schools for researchers. This they are definitely not at present.

For example, facilities cannot be expanded speedily, under the current pressure for places, to achieve overall a better staff-student ratio and to make possible the general adoption of more useful working methods. It should however be feasible for science faculties to introduce research-oriented streams for their third and fourth year students. Entry into these streams would be by merit as judged by the student's performance in the first two years, and the numbers accepted could increase as more facilities become available. In this stream, subjects would be dealt with in depth, and tutorials, dissertations and research projects would replace set practicals and lectures.

Academic staff should be given a

greater role, not indiscriminately, but in recognition of higher qualifications obtained outside the employing department, that is, under present circumstances, in reputable centres abroad. Leave of absence for the purpose should be available.

Furthermore, non-professorial staff should be free, indeed encouraged, to seek, individually or in groups, research grants and thus to participate in government plans to stimulate problem-oriented research.

What has been said is sufficient to explain the annual migration of aspiring students. As undergraduates, they seek a place that was denied them at home. Naturally, most go where entry to a university is—or rather was—an uncomplicated and undemanding business. Thus more than half of them jump from the frying pan of the Greek into the fire of the Italian system. Even Romania has been recently invaded, and is now, like Italy, taking steps to reduce numbers (Table 2).

As far as postgraduate studies are concerned, it would be an excellent thing if Greeks went abroad so as to keep, on their return, national research within the mainstream of world science. In practice, the potential benefit to the country—and to themselves—is reduced by the haphazard acceptance of places anywhere, without due regard to the subject of the research degree, and the suitability of accepting institutions. However, despite its wasteful and unfair character, the process is creating a large pool of people trained in a variety of spheres. It is another matter whether Greece can

make rational use of them.

Reforms

Against this background, the determination of the former and the present governments of Mr K. Karamanlis to reform the educational system becomes better understood.

Compulsory school attendance is being extended from six to nine years, the first six in a primary school and the other three in a junior secondary. In this junior secondary, classics will be studied in modern Greek, thus allowing a shift of emphasis from grammar to content, and making an important saving of time for modern subjects.

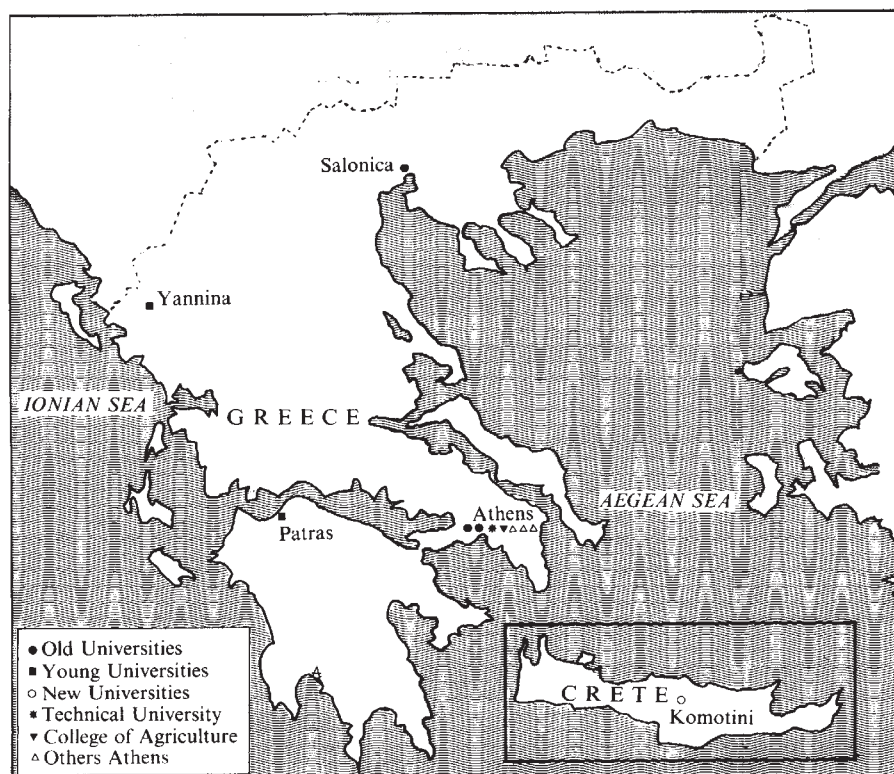
After completion of the junior secondary course, young people will sit examinations for entry to a senior secondary school of either an academic, or a technical-vocational stream. On emerging from the academic secondary, people will be entitled to a university place without entrance examinations (this will take effect as from 1980). Pupils who fail to gain entrance to either type of senior secondary will be enlisted for shorter vocational courses.

Putting such changes into speedy effect is no mean task. Change is proceeding apace, and is accompanied by the appointment of new teachers and the building of new schools at the rate of 1,500 classrooms per year. The demotic form of modern Greek has in the meantime been introduced throughout the educational system. New and better schoolbooks have been produced in record time. Investment of some \$250 million in technical education has just been announced by the government.

At the level of higher education, the young universities of Patras and Yanna are expanding steadily, and two new institutions are being established in Thrace and in Crete. There should, eventually, be sufficient places within the country for most qualified finalists of the senior secondary schools.

Some serious criticism can be made of the way in which the new universities are being set up. Under local pressure, the various faculties of the University of Crete will be scattered throughout the island, making impossible the creation of a coherent campus.

It is also unfortunate that planning of the new universities has been entrusted to detachments of professors from the old institutions. Although some of these people are very distinguished, the risk remains that the new establishments too will be made in the Bavarian style of the old ones. A unique opportunity of introducing creative diversification and innovation into the system will have been missed. Is it too late? □



Canada's nuclear visitor

"THERE are thousands of manmade objects in space. I suppose roughly half of them belong to the Russians and the other half to the Americans. . . . I know there are things up there. I know they are going to fall someday, but I don't want to hear about it until it looks as though they are going to fall in my country. When you hear that, call me again!"

Such was the breezy comment of Canadian Prime Minister Pierre Trudeau on the crash, or, to quote Montreal Radio, "unplanned re-entry" of Kosmos-954. This attitude seems typical of the understatement with which all parties concerned have treated the first cosmonuclear disaster, an attitude which *Pravda* described as "realistic" and "undoubtedly due to the climate of international detente".

Fortunately for the cheer-merchants, the errant satellite descended in an area where, according to Colonel Donald Davidson who led the search team "there is no specific indication" of people save "the odd trapper or trapper . . . a few solitary souls who normally work the area". The specific location of the debris "12.8 km northwest of the landing strip" about 300 km from Baker Lake and 1,500 km northwest of Edmonton brought home to the world at large that Canada had just abandoned the mile. Even the general vicinity of the impact was seized upon by the joke-makers ("Great Slave Lake, eh? Maybe it thought it was heading for the Gulag Archipelago!"). Even the fact that the satellite finally landed in an area with sufficient uranium deposits to give false readings on the search instruments was not without its special brand of irony.

But leaving aside both humour and grim speculation on the devastation which might have resulted had the

satellite fallen further south, a number of questions remain unanswered. Why did debris reach the ground at all, when, according to Tass, the nuclear power unit was designed to be fully destroyed and burnt on re-entry. And,



if the fail-safe construction of the craft was lacking in this respect, would such a burn-up have been, as claimed, without radiation hazard? How safe, indeed, is the debris? (The two members of the search team who found the impact crater have been removed to University Hospital, Edmonton, and the other members to the hospital in Yellowknife.)

More important, what is the procedure for notifying countries that a nuclear satellite may be about to fall? In spite of an opposition hammering, the Canadian Minister of Defence Danson and Secretary of State for External Affairs Jamieson seemed fairly content with the manner in which notification came largely through Washington. However, Canada

is a somewhat special case, being a participant in the US Space Detection and Tracking System via the optical facilities at Cold Lake, Alberta, and St Margarets, New Brunswick. (Canada is also associated with the USA in the NORAD early warning air defence system. Although this relates primarily to manned aircraft attack.) In return for this contribution to the tracking system, Canada automatically receives from the USA all relevant data on space objects. Hence the routing of the warning via Washington is at worst only a slight blow to Canada's *armour propre*. What, however, would have been the procedure if some other target country were involved? Would the message always go via "hotline" to Washington, or perhaps via the nearest "major" power? What then would be, for example, Dublin's reaction to such a warning being routed via London?

International agreements already provide for the search and disposal of crashed space-craft—the craft is to be returned to its owner, the target country reimbursed for damage and search costs. (Will the US participation in the Canadian search cause difficulties here?) What does seem to be lacking is a proper notification procedure, both of spacecraft in distress and spacecraft carrying potentially hazardous material. It would presumably not be impossible to extend the treaty banning nuclear weapons from orbit to nuclear fuel units, or failing an outright ban, to make all such "nuclear" launches notifiable to some central authority. Secretary of State Jamieson and Prime Minister Trudeau have already been discussing possible international talks on the problems arising from the aftermath of Kosmos-954. If these should be forthcoming and fruitful, one could well agree with *Pravda* that this unfortunate incident has, after all, turned out a triumph for detente.

Vera Rich

US agencies to study links between saccharin and cancer

SACCHARIN is to be investigated by the US Food and Drug Administration and the National Cancer Institute, who are to conduct an epidemiological study of 9,000 people in an attempt to determine whether there is a connection between saccharin and bladder cancer.

The dietary habits of 3,000 people diagnosed as having bladder cancer will be compared against those of a randomly-selected 6,000 controls. The study is expected to last 18 months, and to cost \$1.3 million.

Last November, Congress announced a moratorium on proposed legislation that would ban the use of saccharin

because of its suspected link with bladder cancer. Congress also agreed to allocate \$1 million for studies on the safety of saccharin, \$750,000 of which is for the epidemiological survey.

Congress's action followed the publication in October of a report on cancer testing technology and saccharin from its Office of Technology Assessment, which concluded that although there was evidence that saccharin is a potential cause of cancer in humans, there was no reliable quantitative estimates of the risk of saccharin to humans.

According to the OTA report, epidemiological studies of human experience carried out so far have not been sensitive enough to determine if saccharin is a carcinogen when injected. □

NSF encourages more university/industry collaboration

IN a move carefully timed to coincide with the opening of Congressional hearings on its budget application for the year 1979, Dr Richard Atkinson, director of the US National Science Foundation, announced last week that the foundation plans a major expansion of its support for research projects carried out by joint university/industry groups.

No new funds are to be allocated in the NSF budget for such projects: they will be required to compete for support with other regular grants' applications.

Dr Atkinson said, however, that joint projects would be given more active encouragement than in the past, including a major publicity campaign aimed at both industry and universities. Industry was less willing than in the past to risk money on basic research and long-term goals, and was increasingly focusing on product improvement. "Given these trends, the nation is becoming more dependent than ever on universities and colleges to provide the basic research for new technologies and products," he said.

Last week's move follows past criticism from Congress that industry has not been able to compete for research funds on the same basis as university research scientists, a point heavily stressed, for example, by Senator Edward Kennedy during last year's authorisation hearings.

One outcome has been a recent decision by the National Science Board to amend its language covering applications from industry for such grants. Previously it had stated that these would only be considered under certain circumstances, namely if the research was of national importance, or if industry was able to provide unusual expertise or instrumental capability. The new wording is more positive, indicating merely the type of applications that will not—rather than will—be considered.

A particular point stressed by NSF officials is that the delay before pay-off from basic research is a deterrent to industry investing in this area, and that NSF funding might help redress the situation, for example, allowing those scientists who have joined industry because of a lack of jobs in more academic settings to carry out basic science projects.

This argument is not likely to carry much weight with industry, which has recently been telling the government that basic research is a federal responsibility, while development activities should be left to the private sector (a message reflected in the proposed shape of R & D funding within the President's 1979 budget request to Congress).

The sector which is likely to respond much more to the NSF's rallying cry are the small, knowledge-intensive, high-technology firms—such as those distributed around Boston's famous Route 128—many of whom have been experiencing financial difficulties in recent years.

Such firms, however, are rarely interested in basic research as such, even in the long-term. Their main concern—for which there is at present a lack of money—is finding ways of turning a good idea into a marketable product. And this is where, in practice, NSF support will be in greatest demand.

David Dickson

Argentina: US National Academy of Sciences to send delegation

THE Human Rights Committee of the US National Academy of Sciences is to send a small delegation to Argentina next month to investigate charges of repression against scientists.

The delegation, which will also visit Uruguay, will consist of Dr Christian Anfinsen of the National Institution of Arthritis, Metabolic and Digestive Diseases, Nobel Prize Winner in 1972 for work on the structure of proteins, Dr Robert Perry, of the Institute of Cancer Research in Philadelphia, and a member of the NAS staff.

During a ten-day visit to Buenos Aires and Montevideo, the delegation will talk with both scientists and government officials about scientific co-operation with the US, and about the situation of scientists in the two South American countries.

The NAS delegation's visit follows a recent trip to Argentina by Mr Emilio Daddario, president of the American Association for the Advancement of Science, and until recently director of the Office of Technology Assessment.

Mr Daddario said in Washington last week that the actions of the security forces in Argentina had, with a few exceptions, "made a shambles" of scientific freedom, and that reports reaching the West of repression against individual scientists were not exaggerated.

Referring to moves by western scientists to boycott the International Union Against Cancer congress, due to take place in Buenos Aires in October, Mr Daddario said that many of the scientists he had spoken to in Argentina felt it important to maintain contact with their colleagues in other countries, particularly since many of them lacked funds to travel to conferences and meetings abroad.

However he also said that government officials had asked him about the attitude of foreign scientists to the cancer congress, and that he had the impression that they were concerned to appear to maintain normal international relations with the scientific community.

Meanwhile moves are gaining momentum in the US to boycott the congress in protest at the treatment of scientists in Argentina since the army came to power in March 1976. Since that date, many scientists have been reported to have been arrested or abducted without formal charges.

Last month at a meeting of American Cancer Society Research professors in Phoenix, Arizona, all 39 present found that they were unanimous in their individual decisions not to attend the congress if it is held in Argentina as planned.

David Dickson

France falls out with Pakistan over reprocessing

PAKISTAN'S reaction to the latest move of the French government to modify the accord of 1976 on building a nuclear reprocessing plant, has been quite firm and unequivocal. In view of strong public statements there appears little chance that the Pakistani government will succumb to any retraction from the agreement, or soft-peddalling. A Foreign Office spokesman in Islamabad said that the government would not accept any change or modification in the agreement already reached.

The French want to substitute a co-processing plant for the planned plutonium reprocessing plant to guard against separation of plutonium that could be used in explosive devices. A co-processing plant can only reprocess the spent fuel once or twice for use in reactors and it does not produce plutonium. Pakistan's nuclear think-tank, however, has plans for using plutonium, particularly when it develops its fast breeder programme.

Mr Munir Ahmad Khan, the Chairman of PAEC (the Pakistan Atomic

Energy Commission), when asked for the rationale behind the timing of the setting up of the reprocessing plant, explained that PAEC wanted to synchronise the installation of the reprocessing plant with the commissioning of a few more reactors, but that for various reasons the reactor programme is running behind schedule. Mr Munir Ahmad explained, however, that the reprocessing technology is highly sophisticated and takes longer to set up than a reactor.

His team would like enough time to acquire the necessary experience in reprocessing technology for its optimum use. Hence, the hurry for the reprocessing plant.

At Chashma, the site of the proposed plant (plus a 600 MW reactor), the foundation concreting is in progress in anticipation of things ultimately working out as planned.

In his recent address to the Institute of International Affairs in Karachi, Mr Munir Ahmad explained the technical and economic reasons for building a

reprocessing plant in a country like Pakistan—which is planning on deriving almost half its energy supply from nuclear sources by the end of the century. Even the 2,000 MW from the big earth-filled Tarbela dam, he claimed, would be insignificant in filling the country's energy gap. And oil-fired power stations were becoming less viable, especially as Pakistan had so far struck almost no oil or good quality coal. So, the only option, according to Mr Munir Ahmad, would be the nuclear one.

His view is that in the overall nuclear fuel economy framework, reprocessing of used fuel plus plutonium is inescapable. Jacques Couture, sales manager of the reprocessing division of the French company, Compagnie Generale des Matiere Nucleaires, has not missed his opportunity to blow the re-

processing trumpet. He has said that "with a typical light-water reactor which needs about 180 tonnes of natural uranium a year, the annual saving will be at least 40 tonnes, or even 70 tonnes if the plutonium recovered during reprocessing as well as the uranium is recycled." In fast breeder reactors, the fuel is some 20% plutonium oxide in uranium oxide, and the nuclear expert's view here is that a co-processing plant that cannot deliver plutonium is, therefore, not of much value, seen in that context.

Mr Munir Ahmad was vehement in his address on the topic of proliferation. He said that the safeguard imposed by IAEA had been fully incorporated in the agreement and that the French had imposed some additional safeguards. He saw no valid reason for the scepticism which had

been shown, and thought it might not be unfair to conclude that the motive for changing the agreement was to deny a developing country a useful, sophisticated technology of future.

Pakistan has sizable deposits of uranium and thorium-bearing minerals. Pakistani scientists are exploring the possibility of exploiting the indigenous sources of thorium in a thorium-uranium-233 cycle in fast breeder reactors, for which plutonium is essential.

The French have developed a proliferation-free uranium enrichment process—a chemical exchange method—but it is unlikely to be commercially viable for another decade or so. Likewise, the French concept of the co-processing plant has yet to be commercially accepted as it has not been tried in any other country.

Azim Kidwai

Europe's nuclear circus

PLENTY of shadow-boxing went on at last week's open discussions on nuclear energy organised by the European Commission, with no side quite hitting home but nevertheless a lot of energy expended. The proceedings were launched by news of an accident at one of Belgium's nuclear plants on 13 January. The accident was made public by the Environmental Protection Society who claimed that 80 people were contaminated by radium-131 and that the affair had been covered up by government authorities.

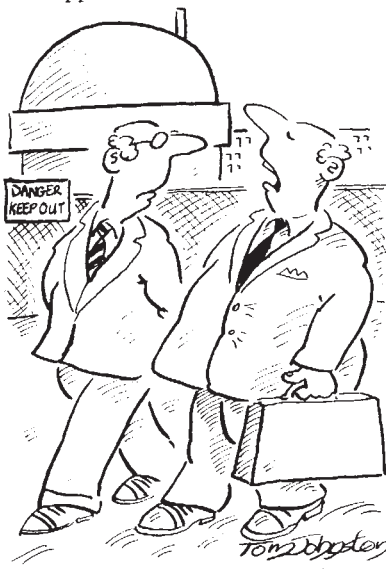
The news caused quite a scare in Belgium, but was ridiculed by speakers at the nuclear debate, doctors, and the director of the Tihange plant near Liège, where the leak occurred.

The so-called 'father of the H-bomb', Dr Edward Teller, a leading US nuclear physicist at the Hoover Institute for War, Revolution, and Peace, Stanford, California, told the nuclear hearings panel and an audience of several hundred that he was 'more alarmed' at the kidnapping of Baron Edouard Empain in Paris by terrorists, than by the Belgian leak. The Hungarian-born scientist claimed that he had been exposed to more radiation when he flew over Europe to be present at the hearings.

The state-run electricity company, responsible for the Tihange plant, put the figure of people involved at between 30 and 60. Director Robert Van den Damme said that no members of the public were involved during the accident which happened during an annual maintenance check. He said that workers ignored the leak until the job was done. Six people had

to be examined by doctors, who they said had been contaminated by 100 millirems of radium-131. They added that the maximum tolerated dose was 3,000 millirems for 13 weeks before the possibility of thyroid cancer occurred.

The Friends of the Earth argued that the principal issue was one of disclosure; that the authorities should have made the accident public and that their natural reaction had been to conceal it. But Mr Van den Damme denied that the affair had been hushed up. He said that the leak had not escaped from the plant and had therefore done no damage to the environment or the surrounding population. "It was not worthwhile to publish information about something that did not happen".



"If there is a leak, let's hope there are some terrorists in the area!"

The news did manage to give the Commission's nuclear hearings more relevance. The session ended after three days of heated debate between the supporters of nuclear energy and its critics. The highlight for many was described as a "confrontation" between two old debating partners, Robert Jungk, an Austrian futurologist opposed to the use of nuclear energy, and the controversial Dr Teller.

The theme of the session 'Economic growth and energy options: implications for safety, health and environmental protection', considered the problems of terrorist activities and the proliferation of nuclear weapons. Jungk argued that increased security measures to guard against the two problems could lead to centralisation and therefore police states. He said that this was a form of mental pollution that was not acceptable and advocated the immediate suspension of the nuclear energy programme.

Dr Teller hit back by describing what he termed as the worst possible pollution, that of poverty. He argued that the developed countries should devote their energy to developing nuclear power. He said that oil should be given to the third world to enable it to develop, whereas nuclear power was economically and structurally beyond its reach. And he put paid to terrorists by theatrically declaring that "the only way to get rid of terrorism is to get rid of terrorists".

The debate at least guaranteed a full house. EEC energy commissioner Guido Brunner made no final assessment of the findings of the hearings. Critics claim that there was never an intention of altering the Commission's approach to energy policy as a result of the debate.

Patricia Kelly

Polish censorship

COPIES of the Polish censorship regulations and instructions for the period February 1974–February 1977 have recently become available, via the unofficial “Social Self-Defence Committee” (formerly the “Workers’ Defence Committee”).

The subjects of banned articles during this period included: reports on industrial pollution of the environment, criticism of the bill on psychiatric medicine, information on the carcinogenic properties of PVC, discussion of the backwardness of scientific education in comparison with the West, errors in the selection of candidates for higher education, and data on the type of parasites afflicting Polish cattle.

The standing “Notes and Recommendations of the Central Office of Control of the Press, Publications and Entertainment” specifically prohibited publication of information on chemical hazards in industry and agriculture, work safety and hygiene, alcoholism, the Katowice mine disaster, and the purchase by Poland of production licences from capitalist countries.

Vera Rich

US soldiers in cancer study

THE US Department of Defense has promised to undertake a “crash programme” to examine the records of military personnel exposed to nuclear weapons tests in the 1950s to see if they provide evidence of any abnormal incidence of cancer. The promise was made by the deputy director of the Defense Nuclear Agency, to a House sub-committee on health and the environment during the course of public hearings last week on the dangers of low level radiation.

The sub-committee has been particularly concerned at evidence suggesting an abnormally high incidence of leukaemia among the 2,235 soldiers who took part in manoeuvres after a 45-kiloton test shot, known as Smokey, which was detonated in the Nevada desert in 1957.

A study of the possible carcinogenic effects of radiation on those involved is being carried out by the Center for Disease Control, whose director, Dr William H. Foege, told the sub-committee that the eight reported cases of leukaemia among the 2,235 was “out of the normal range”.

Earlier Dr Karl Z. Morgan, who at the time of the test shot was director of health physics at the Atomic Energy Commission’s Oak Ridge Laboratory, told the committee that his staff had been unable to retrieve scientific measuring equipment after

the shot because of “residual contamination”. He had “no doubt whatever” that this radiation had caused the leukaemia now found in those who had taken part in the manoeuvres.

Dr Morgan, who is now a professor at the school of nuclear engineering of the Georgia Institute of Technology, criticised scientists who refused to accept evidence that there is no safe level of radiation, and that any dose, however small, can cause leukaemia and other forms of cancer.

“One of the many problems we face today is that many scientists have accepted the threshold hypothesis as a cardinal law and have lived with this hypothesis so long that they have become staid or petrified in their thinking. Now they cannot believe or accept the fact that the threshold hypothesis is wrong”, Dr Morgan said.

David Dickson

Brothers in human rights test case

THE cyberneticist Gol’dshstein brothers—Grigorii and Isai—of Tbilisi, first hit the “human rights” headlines in 1975, when their exclusion from the Tbilisi Conference on Artificial Intelligence led to a confrontation between the international conference committee, who urged their admission, and the Georgian “hosts”. The brothers are now, once again, involved in what may prove to be a test case—Grigorii has been arrested, and Isai has been threatened with arrest on the charge of parasitism and “malicious evasion of socially useful work”.

The charge of “parasitism” (being without visible means of support) was brought last year against refusnik Iosif Begun, who was sentenced to two years exile in Siberia. The authorities refused to acknowledge that he had

any means of livelihood, since his current occupation—teaching Hebrew—was not a recognised profession. The Gol’dshstein bothers, however, until their dismissal from the Mendelev Institute of Metrology in 1971, had been earning 600 roubles (£300) per month each. Since the minimum wage in the Soviet Union is 60 roubles per month, the Gol’dshsteins have managed to convince the authorities that they have been living on savings lawfully laid by from their earnings. Nevertheless, it is claimed, they are evading their responsibilities by failing to undertake socially useful work.

Both brothers were dismissed from their Institute in the routine manner, following their application for an emigration visa—an application which was refused on the familiar grounds of access to secret information. Unlike most refusniks, they have now been “offered” jobs commensurate with their academic status, Grigorii in October 1977 and Isai in January 1978. These jobs, however, are at the “closed” Institute of Explosives Research in Tbilisi, where all work is potentially secret, and both in turn have refused to accept the offers.

On 17 January, Grigorii was arrested, and was released ten days later on a written undertaking not to leave Tbilisi. His trial is expected “perhaps in two to three weeks”. On 19 January, Isai received an ultimatum from the police, that unless he finds a job within a month, he too will be arrested.

The Gol’dshstein brothers are so adamant in their desire to emigrate to Israel that in 1972 they unilaterally renounced their Soviet citizenship. Clearly, they are hardly the loyal comrades one would expect to find working in a secret institution. The jobs proposed for them are, they feel, yet another means of preventing their emigration.

Vera Rich

New threat to helium reserves

A demand that “all appropriate measures” be taken to conserve rapidly-diminishing supplies of helium for potential future use has been made in a report published last week by a committee of the US National Academy of Sciences. The committee, which was set up last autumn to provide the administration with independent advice on the politically-sensitive issue of helium conservation, suggests that there is “a strong case for building a substantial government-owned strategic reserve of helium”. It also condemns the present venting of separated helium into the atmosphere by natural gas-producers.

Most scientists know helium merely as the second lightest element. Some have used its properties as a gas to fill meteorological balloons; others have more recently exploited its superconducting properties at temperatures close to absolute zero—where it remains in liquid form—for experiments ranging from nuclear fusion to the superconducting magnets that will be used in Fermilab’s new energy saver/doubler.

But although current applications remain relatively limited, helium has become a major headache for the US government. Reserves of helium-rich natural gas, from which helium can

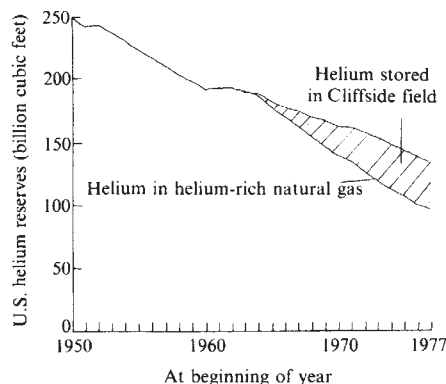
be extracted many times more cheaply than from air, are known to be very limited and, at current extraction rates, rapidly diminishing.

Awareness of a potential shortage of helium dates back to 1960 when the administration, with one eye on future federal needs for both defence and space research projects, passed a Helium Act. Under the terms of the act four companies were contracted to separate helium for storage from natural gas; the conserved helium was stored in the partially depleted Cliffside natural gas field near Amarillo in Texas, which at present contains about 39 billion cubic feet (bcf) of the gas.

But the act turned out to be an embarrassing and expensive mistake. With the run-down in both defence research and the space programme at the end of the 1960s, demand for helium dropped dramatically below expected levels—by 1971 the total market demand for helium was only half what had been predicted—with no indications that the situation would improve in the near future. The government's problems were compounded by the fact that private suppliers were able to offer helium at much lower prices than the administration.

The net result was that sales of helium by the Bureau of Mines in 1971 were lower by a factor of six than had been predicted. And the massive federal investment in conservation measures had resulted in an annual bill in interest charges alone of over \$20 million, with sales averaging only about \$1 million. One reaction was the immediate termination of the contracts with the four private suppliers—action which has resulted in lengthy litigation still making its way through the courts. Since then, natural gas producers have been venting helium either directly or indirectly into the atmosphere. And there have been growing demands from Congress that the whole issue of helium conservation should be looked at more closely, with an eye to new legislation replacing the 1960 act.

Last September the appropriation committees of both the Senate and the House ordered that a study of the present situation, with recommendations about possible new legislation, be carried out jointly by the Department of the Interior's Bureau of Mines and the Department of Energy (then the Energy Research and Development Administration). In their turn, these two agencies, which had also brought in the Department of Defense and the National Aeronautics and Space Administration (NASA) as two of the largest helium-users, decided that an assessment of the situation should be obtained from an independent non-



government organisation, namely the National Academy of Sciences.

Last week's report is the result of this study, carried out in only a few months by a specially constituted committee of the National Research Council (and taking into account the results of a public hearing on helium conservation measures held in Washington in November).

The committee's report is unequivocal and, at times, outspoken. "Obscured from public view by current dilemmas associated with energy supply and an unstable economy, public policy for the management of the nation's resources appears inadequate and inflexible" it says.

The committee points out that almost 96% of the world's resources of helium-rich natural gas is to be found in the US, and that with current rates of extraction, these are expected to run out within 20 or 30 years. Furthermore although current annual demand is only about 1 bcf, this figure is likely to be considerably higher by the middle of the next century, with potential applications in fields such as fusion and gas nuclear reactors, laser-based missile defence systems, and advanced energy conversion cycle.

"It is clear that even the maximum possible helium conservation programme will only buy time in which to adjust to the extraction of helium from air as the sole source" the committee says. It agrees that as a result of earlier conservation policies there is at present enough helium stored to meet federal requirements into the next century, but writes that "the case for helium conservation is long-term based on the interest of future generations . . ."

The report also points out that there is, at present, little market incentive for conservation of helium by private producers. It therefore suggests that the government contemplate taking "an aggressive role to resolve the legal and market uncertainties currently discouraging helium conservation."

Although the conclusions of the NAS report coincide broadly with the views of the government agencies

which commissioned it, they are likely to put a more subdued case in their own report to Congress, which is expected to be delivered within the next few weeks. Administration officials agree, for example, on the need to stimulate private industry's interest in helium conservation—but they feel that by concentrating on the the situation of supplies of helium-rich gas, insufficient attention has been given to the potential for extracting helium from ordinary natural gas.

Furthermore there is a feeling that the NAS report may have been too optimistic in its predictions of future demand for helium. For example, the report quotes a prediction by the Argonne National Laboratory that by the year 2050 the annual requirement for helium from fusion reactors and superconducting energy storage alone will be between 10.4 and 15.4 bcf. In contrast, information gathered from government agencies indicate that consumption in 2030 will probably be between 3 and 8 bcf.

The agencies, which have been asked by Congress for advice as to possible government action and future legislation, are therefore unlikely to recommend the renewed stockpiling of helium, at least not in the near future. Dr Ray Munnerlyn, who is responsible for helium policy at the Department of the Interior, said last week that in the present circumstances, he would personally be opposed to a recommendation that Congress should renew its helium purchases.

David Dickson

Erratum

We would like to apologise for the errors which slipped into the article 'Dioxin meeting recommends cancer study' on page 202 of 19 January issue. They resulted from dictation over the phone and subsequent editing and are amended as follows. The first sentence of the first paragraph should have begun: "Since the release of the tetrachlorinated dibenzo-p-dioxin (the isomer 2,3,7,8 tetrachlorodibenzo-p-dioxin) . . ." The company referred to as the Doll Chemical Company is in fact the Dow Chemical Company and Dr Rodolfo Saracci is at the Unit of Epidemiology and Biostatistics, not Immunology and Biostatistics as reported. The two groups from the University of Wisconsin and the Dow Chemical Company, which the article claimed reported animal studies to test the carcinogenicity of dioxin, were not in fact present at the meeting; their work was discussed in their absence.

correspondence

Catastrophe theory reply

SIR,—This is a reply to a number of recent letters (*Nature* 270, 381–384 and 658; 1977) attacking our article (Zahler & Sussmann *Nature* 269, 759; 1977) on applied catastrophe theory. Many of these cite applications in the physical sciences to rebut our criticism; but as we were careful to state repeatedly in our paper, our discussion applied only to models in the biological and social sciences. In a more specific complaint, the respected mathematician John Guckenheimer asserts that we ignored recent work of Kozak and Benham on denaturation (Benham & Kozak *J. Theor. Bio.* 63, 125; 1976), where the Maxwell convention, not the delay rule, is used. The effect of this change, however, is to remove the cusp, and thus the catastrophe theory, from the analysis. Dodson's letter gives another reason why catastrophe theory should not apply to denaturation.

A number of writers defend Zeeman's embryology paper (Zeeman *Lectures on Mathematics in the Life Sciences*, 7, 69; 1974). The meaninglessness of this paper's "first-order" quantitative results will be explained in Sussmann & Zahler *Synthese* (in the press). Guckenheimer complains that our discussion of evidence is misleading. We printed Zeeman's statement verbatim and then five facts which no one has disputed (with one minor exception; see below). Despite Guckenheimer's attempt somehow to separate confirmation of a model from confirmation of that model's predictions, we feel that most readers will agree that our assessment, and not Zeeman's, is supported by the facts.

Zeeman writes that our article ignores a rigorous version of his main theorem published elsewhere (Zeeman *Proc. Int. Cong. Math. Vancouver* 2, 533; 1974). We criticised the first proof for unjustifiably singling out the time-axis. The second proof adds an assumption, not mentioned in the paper addressed to biologists, which declares that the time-axis is special—postulating what cannot be proved. Ignoring all the other faults we found in his proof (which are not repaired by the new assumption), Zeeman concentrates his defences on one of our minor criticisms, which concerns the direction of curl of the isolated neural plate. In rebuttal we note that it is Zeeman, not ourselves, who is misquoting Crelin, who clearly states that the graft curls

laterally (Crelin *J. Exp. Zool.* 120, 547; 1952 and private communication); that the mesoderm is too thin to exert the forces Zeeman ascribes to it; and that 'neurulation', the subject of the section containing Zeeman's statement, includes Harrison stage 23, which Crelin studied.

Returning to Prof Guckenheimer's letter: he disagrees with our definition of the word 'catastrophe'; but the definition we use is that used by all the applied catastrophe theorists that Guckenheimer is defending. We agree with Guckenheimer's statement that the confusion of the intuitive notion of jump with the mathematical notion of jump discontinuity is common and useful. What we object to are attempts to switch from one to the other and back in mid-proof. Finally, Guckenheimer complains that our remark about the large number of unrefereed applied catastrophe theory papers is "snide". First of all, it is a fact. Consider, for example, the bibliography prepared by the strong catastrophe-theory supporter Lynn Steen in February, 1977 (Steen: *Catastrophe Theory: A Selected Bibliography* (mimeographed, 1977)). Approximately 70% of the papers classified there as applied catastrophe theory appear to be unrefereed; we think that this is an unusually high ratio for scientific papers. While no one would say that it is wrong to publish an unrefereed paper, we think it is quite proper to point out this phenomenon, because it indicates that catastrophe theory has grown in an atmosphere largely sheltered from outside criticism, and this partly accounts for its exuberant claims.

RAPHAEL S. ZAHLER
New Haven, Connecticut

In support of boycotts

SIR,—Richard Peto and Sir Richard Doll raise an important and difficult issue in their letter about boycotts of congresses to be held in countries controlled by repressive regimes (1 December, page 384). As one who lived and worked in South Africa until government action forced me to leave, I can say something about this topic from 'the inside', as it was a subject often discussed by those of us who wished to see change and about which conflicting views were held. I emerged from these discussions as a strong proponent of boycotts for the following

reasons (most of which form answers to the points raised by Peto and Doll):

- It is often argued that boycotts cause harm to the people one least wishes to harm—opponents of the regime, scientists who need "contact with the international community" or, in the case of economic boycotts, the mass of people of the country (for example, the blacks of South Africa). The fallacy in that argument is that most genuine opponents of repressive regimes will already have been dealt with by the government—imprisoned, silenced by banning, or even murdered. In most countries scientists can, and do keep contact with the rest of the world by travel and are not restricted unless they are actively engaged in politics or openly express opposition to the regime; if they do, they are likely to be dealt with as above. And, in my experience, a majority of politically-aware blacks in South Africa would have accepted the damage that might have resulted from economic sanctions as a necessary and effective means of putting pressure on the government.

- Visits to these countries by prominent people or holding of international congresses is often regarded as tacit acceptance of the regime by the rulers and sometimes exploited as evidence of its acceptability. To opponents of the regime such actions bring a sense of disillusionment and cynicism, and I recall the moral encouragement and gratification one felt whenever a prominent artist, scientist or sportsman announced publicly that he would not visit South Africa.

- Peto and Doll imply that scientists should be "apolitical" as this confers on them a special "useful image", presumably to be used for protests, such as that made by so many scientists against the imprisonment of Mikhail Shtern. The argument may be used with equal force by doctors, sportsmen, artists, businessmen or any other person pursuing any other occupation! No group is immune from the responsibility to take a political stance at times, whether this is expressed as a letter of protest or a boycott.

- Boycotts are often said to be ineffective and therefore not worth supporting. This is only true when they are not fully supported. The sporting isolation of South Africa has been most effective and has led to removal of many of the barriers imposed in that country against mixed-

race sporting activities.

● Would-be visitors to these countries often say they go to see and hear things for themselves. Indeed, I have often been asked to provide introductions to black opponents of South Africa's regime who could provide visitors with their side of the story. I reply that most genuine opponents have already been silenced one way or another and I would certainly not put at risk those who had escaped the net of repression by encouraging them to speak out to inquisitive strangers. I have known many doctors and scientists who have visited South Africa in the past ten years; most return to this country with new arguments and convictions, if not fully in favour of apartheid, at least condoning or "understanding" it, now that they've "seen the problem" for themselves (or been indoctrinated by their proselytising hosts?). Some come back encouraged by a few whispered sentiments of distaste for the regime, mistakenly to believe that there still exists in that country a substantial courageous body of doctors or scientists who oppose apartheid and are doing something actively about it. Not one of my colleagues has met a black person socially, and none has spoken out against the regime while he was there ("How could I say anything when they were my hosts?").

Clearly, one could carry such convictions to absurd lengths. Some degree of repression exists in most countries; minority groups often appear to be singled out and treated badly. Where one draws the line is a matter for individual decision. What I'm saying is simply that if one feels strongly enough about a situation in a particular country, there are good arguments in favour of staying away—and of making one's reasons known.

R. HOFFENBERG

The University of Birmingham, UK

Sauce bearnaise

SIR,—Perram, Nicolau and Perram recently described a method for reversing what they called coagulation of *sauce béarnaise* by addition of extra vinegar (*Nature* **270**, 572–573; 1977). They stated that other authors recommend discontinuation of the 'experiment' of producing *sauce béarnaise* when coagulation occurs.

Amateur, non-mathematical cooks, such as myself, know that two kinds of deviation from homogeneity can occur during the preparation of *sauce béarnaise* and similar sauces such as *sauce hollandaise* (see Elizabeth David's book, *Summer Cooking*, first published by Penguin in 1955). The colloidal suspension can separate into its components, no doubt due to the forces

described by Perram *et al.* I have found, however, that sauce which has failed in this way can be made homogeneous quite simply by the addition of a few drops of warm water with steady stirring. This has the advantage of maintaining the delicate flavour balance, which would be disturbed by the unnecessary addition of extra vinegar. The second deviation from homogeneity occurs when the temperature of the sauce rises too high: the egg yolk becomes coagulated—as Elizabeth David notes—and the sauce becomes grainy in texture. This process cannot be reversed by the addition of acetic acid and it is to this event which culinary authors are referring when they recommend discontinuation of the 'experiment'.

ROY JOHNSON

Cambridge, UK

SIR,—We welcomed the long overdue treatise by Perram *et al.* on the stability of the colloidal suspension colloquially known as *sauce bearnaise*. However, as the authors claim primacy in reporting their successful attempt to resurrect this sauce by employing the theory of the stability of lyophobic colloids, we feel compelled to mention a previous notation in this field—Child, J., Bertholle, L. & Beck, S. *Mastering the Art of French Cooking* (Knopf, New York, 1966). The use of *lemon juice* here substitutes for acetic acid.

G. STEARNS

R. WEININGER

New Haven, Connecticut

SIR,—A professional biologist (and amateur cook) is amazed that Perram, Nicolau and Perram could by consideration of the theory of stability of lyophobic colloids deduce that lumpy *sauce béarnaise* could be made smooth by the addition of acetic acid and confirm it in practice. The biologist would not call the lumpiness coagulation, which to him is an irreversible reaction (Chambers Dictionary of Science and Technology) though the chemist may. The biologist would also control his experiment by addition of acetic acid solution without the acetic acid, i.e. water. This, reducing ionic strength, the practical cook has done with equal success for decades, though this 'know-how' is seldom described in manuals of gastronomy.

Furthermore, the first step in preparation of *sauce béarnaise* is a drastic 'reduction' by heat. Does not this remove most, if not all, of the original volatile acetic acid molecules before the addition of the fats? If so, the charge on the fat droplets must be provided not by acetic acid molecules, as postulated, but by less volatile acids

of the herbs and vinegar—impurities to the chemist but quintessential factors to the gourmet and variable damn-nuances to the cook.

J. F. LOUTIT

MRC Radiobiology Unit,
Harwell, UK

SIR,—Perram *et al.* are to be congratulated on their attempt to define the underlying physico-chemical principles of the unstable system known as *sauce béarnaise*, and in particular on their published observation that addition of vinegar (acetic acid) will resurrect the sauce.

However, I must point out that my wife has adopted this practice for some years now on the very rare occasions when the sauce has coagulated. No doubt, other good cooks have done the same: cooks, however, unlike scientists, usually prefer to keep their knowhow secret rather than divulge it.

T. S. TWEEDIE

Chester, UK

Calculating the fine structure constant

SIR,—I would like to cite two references *Physics Today*, **24**, 8, 17 (1971) and **24**, 11, 9 (1971), in which reports have been published of attempts to calculate empirically the fine structure constant, whose experimental value in physics is 137.03602.

In these papers, the authors have attempted to calculate the value of the constant with pure members and π . The results are all very complex, and none are in exact agreement with the experiment.

I would like to propose a much simpler solution whose value is in exact agreement with the experiment.

$$137.0360157 = [137^2 + \pi^2]^{1/2}$$

Since this expression is very simple, it must be the true solution.

THOMAS J. BURGER

3M Company, Minnesota

Bright minds not wasted

SIR,—Your editorial (24 November, page 287) on the dearth of scientific appointments was immodest, probably unintentionally. "How do we get ourselves out of this bind, ensuring that bright minds are not wasted?", is the sentence that offends. I for one do not feel that my students will be necessarily accepting second-best if they do not become professional scientists.

The tenor of the article was antagonistic to the idea of the best minds going into other fields. Is it not this attitude which has made it necessary to force such a change for the good of the larger community?

S. D. DOVER

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news and views

Chemical waves in *Drosophila*

from Jonathan Slack

OTHER sciences may progress by conjecture and refutation but this can hardly be said of contemporary developmental biology. Here progress occurs by the successive formulation of models whose quality is judged, at least in the first instance, by the amount of applause from the audience when they are first unveiled.

1978 has started well with a new model from S. Kauffman, R. Shymko and K. Trabert with what amounts to a general theory of development in the fruit fly *Drosophila melanogaster* (*Science* **199**, 259; 1978). The new model does nothing less than account for the relative positions of different parts of the organism and the assignment of the correct intrinsic characteristics of each part.

To understand what this means we must remember that most of the adult fly is formed during metamorphosis from groups of cells called imaginal disks. The rudiments of these disks are laid down in early embryonic development and during the life of the larva they grow but do not visibly differentiate. It has recently been shown that during their growth the disks become subdivided into compartments (Garcia-Bellido, Ripoll & Morata *Devl Biol.* **48**, 132; 1976). These compartments have the property that once the boundary has formed, the cells from the neighbouring compartments can never cross it, so each develops by further subdivision but not by recruitment of cells from outside. Certain homeotic mutations result in the partial or total conversion of one compartment within a disk into another. For example, the mutant *engrailed* converts the posterior compartment of each of the thoracic structures (wing, legs, haltere) into the anterior compartment. (Lawrence & Morata *Devl Biol.* **50**, 321; 1976). It is now widely believed that the compartment boundaries are the thresholds between the on and off states of the wild type alleles of what Garcia-Bellido calls selector genes; in which occur the homeotic mutations.

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Another property of the imaginal disks is that they can be cultured in the abdomens of adult flies for long periods of time and then forced to differentiate by implantation into a larva about to undergo metamorphosis. In general their state of determination (leg, wing, genital, and so on) is conserved during culture, but occasionally a so-called transdetermination occurs whereby a certain type of disk changes to another state of determination. Several of these changes are also mimicked *in vivo* by homeotic mutations.

Kauffman has previously published a most persuasive analysis of this and related data from which he concluded that the states of determination of the disks are given by a combinatorial 'epigenetic code' of 'on' and 'off' states of the selector genes (*Science* **181**, 310; 1973). A mutant selector gene will behave as though it were off in any combination and so may change several disks or compartments to different states of determination.

So, the development of the adult fly involves first the regionalisation of the embryo into a number of territories which will become the various disks and perhaps also the larval segments, and second a subdivision of each of the disks into its compartments. We wish to know how these subdivisions can be carried out to yield the correct combinations of selector gene activity in each compartment, and how the positions of the compartment boundaries, or thresholds of gene activity, are determined. The new model proposes an answer to these questions and it does so by bringing a new actor onto the stage to join the gradients and thresholds which usually play out the drama on their own.

Dissipative structures

This is the dissipative structure, the theory of which has been developed over the past 30 years by the able team of physical chemists in Brussels (see *Self Organisation in Nonequilibrium Systems*, Nicholis G. & Prigogine I., 1977). A spatial dissipative structure is a stable pattern of chemical concentrations distributed in space.

Such phenomena are called 'dissipative' because they can only arise in systems which are maintained far from thermodynamic equilibrium by a continuous flux of matter through the network of reactions.

This situation may seldom be encountered in the test tube but is of course the norm for biochemical reactions in the living cell. Dissipative structures are possible for coupled pairs of reactions which have particular relations of auto and cross catalysis, and although most of the work on their properties has been theoretical, several of the predicted dynamical features are realised in the Belousov-Zhabotinskii reaction (Winfree *Science* **175**, 634; 1972).

Drosophila model

As far as the *Drosophila* model is concerned the essential feature of this type of reaction network is that many dissipative structures may be possible for the same system. As a controlling parameter is varied the system will pass through a predictable sequence of spatial patterns. The conditions for the transitions can be established by the mathematical techniques of bifurcation theory which can indicate the critical parameter values at which the homogeneous state ceases to be stable to thermal noise, and which particular patterns present in the thermal noise will be selected for amplification.

Consider a straightforward example where the reactions are proceeding in a tube of length L . If L is small enough only the homogeneous state will be stable. Now imagine that the length is gradually increased. Above a critical value L_1 , the homogeneous state will cease to be stable and will be replaced by a monotonic gradient. At a greater length L_2 , the homogeneous state will be re-established. At L_3 , a second pattern mode will appear, which is a distribution of concentration of $\wedge$ or $\vee$ form. At L_4 , the second mode will revert to homogeneity. At L_5 , a third mode will arise, of the form $\sim$, and so on. In the general case there may not be a unique final outcome for a given length, but in the *Drosophila*

model there is a separate interval of length for each successive mode and the rather deep mathematical problem of predicting intermode transitions is avoided.

Suppose now that the reactions are proceeding not in a tube but in a growing line of cells. The cells contain switches which will turn on the selector genes when the concentration of the dissipative structure reagent exceeds a certain value. We have to assume that a different switch becomes responsive each time a new pattern mode is established. We further assume that the threshold values for all the switches are near the nodal values of the patterns, that is the concentrations which are the same as those of the homogeneous steady states. So when the first mode is set up with a single node in the centre, the first selector gene is turned on in one half of the line of cells and off in the other half. When the second mode is established there are two nodes which bisect the two regions defined by the first switch. If the first modes are of the respective form $\backslash$ and $\swarrow$, then the states of gene activity from left to right will be 11, 10, 00, 01. As each new mode appears, its switch will bisect previous regions in such a way that each new compartment is defined by a unique combination of states of gene activity.

This is the essence of the *Drosophila* model. The patterns are more complicated because they are formed not in a line of cells but on various two-dimensional domains: a growing ellipse

represents the growing imaginal disks and an ellipsoidal surface represents the early embryo. It is shown that the nodal lines which appear on an expanding ellipse are very similar to the arrangement of observed compartment boundaries on the fate map of an imaginal disk. The early embryo does not grow in size, but here a fall in diffusion constants due to cellularisation could have an analogous role. It is proposed that five sets of boundaries arise and divide the embryo into 32 regions. The binary coding of the selector genes in these regions corresponds very closely to that predicted from relative transdetermination frequencies, and to the occurrence of certain homeotic mutations.

Is it correct?

If the model were even approximately correct, it would certainly go down in history as a great scientific advance. Its beauty derives from the diverse strands of thinking which come together in the one great synthesis: gradients, thresholds, a binary combinatorial epigenetic code, dissipative structures.

But is it right? In its favour are several impressive retrodictions of known facts. For example, it is known that the anteroposterior subdivision of the embryo commences before the dorsoventral. So the model can lay down both axes of the body with the one mechanism, and the sequence arises naturally from the fact that the long axis of an ellipsoid is longer than

the short axis. Another striking example is the mutant *antennapedia* which converts the antenna into the second leg. The model explains that it must be the second leg rather than the first or third because of their different specific codings.

There are also several known facts which seem unfavourable for the model at the moment. It would be churlish to run through a long catalogue of counter arguments, but one group of phenomena is so strongly at variance that it cannot be ignored. This is the evidence for some sort of monotonic gradient which controls pattern formation in the early embryo. The evidence for a single (or double) controlling gradient is discussed in detail by Sander (*Adv. Insect Physiol.* **12**, 152; 1976) and rests largely on the results of constriction experiments on the eggs of a variety of insect species. There is also evidence for the existence of a persistent monotonic gradient in the imaginal disks which enables fragments to regenerate, sometimes across the compartment boundaries (Bryant *Curr. Top. Devl Biol.* **8**, 41; 1974). The observed behaviours in these experiments are hard to explain by the new model. It should also be borne in mind that the evidence for gradients exists in many developmental systems which have been carefully examined (sea urchin, *Hydra*, and the vertebrate limb for example) while the evidence for binary decisions and a combinatorial code, persuasive as it is, is confined to the insects alone. □

Replication origins

from David Sherratt

A REPLICON is a contiguous segment of DNA constituting a unit of replication. By definition a replicon controls its own replication; determination of the rate of DNA initiation from a unique origin is generally accepted as the point of control. Most information about origins, initiation and subsequent movement of the replication fork has been furnished by simple viral and plasmid replicons. However, because these depend on their hosts for most of the DNA replication machinery, it should be possible to extrapolate to the more complex host chromosomes.

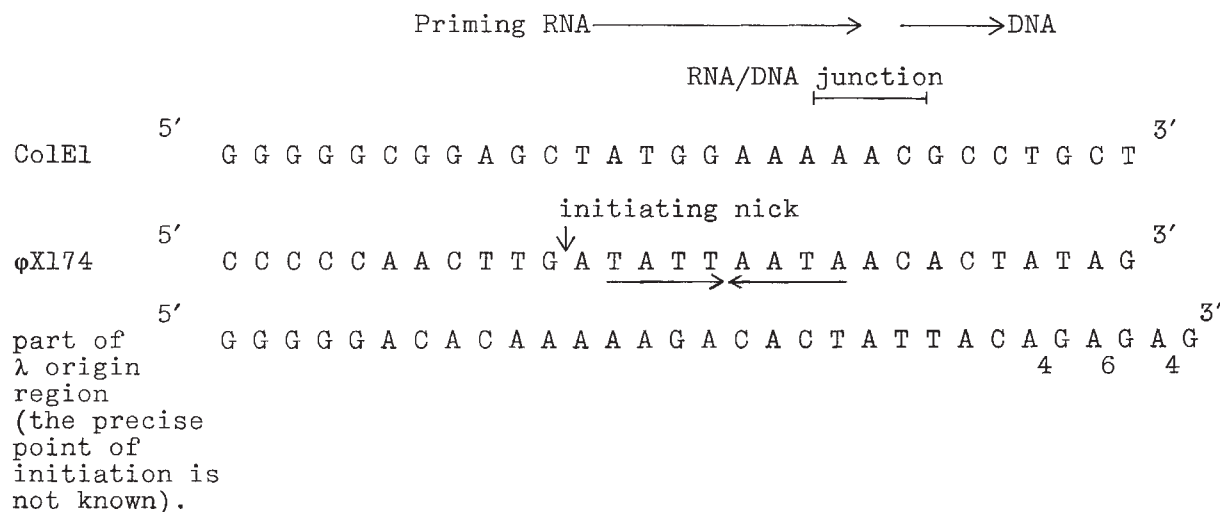
DNA polymerases, unlike RNA polymerases, are unable to initiate polynucleotide chains *de novo*, probably because of the high probability of introducing irrevocable error by mispolymerisation on a short chain, which by

its nature is poorly base-paired to the template strand. Two ways of overcoming this dilemma are known. The most widespread is to utilise RNA 'primers' to provide a base-paired 3'OH group to which deoxyribonucleotides can be added. Since this RNA is 'non-genetic' and is later removed, errors in its polymerisation can be tolerated. Unidirectional or bidirectional replication can follow equally well from such RNA priming. In principle, this type of initiation could be controlled either at the level of initiation of RNA synthesis or at RNA termination/DNA initiation.

Another apparently less common way of initiating DNA replication is to create a deoxyribonucleotide 3'OH primer by nicking a parental DNA strand internally. Replication from such a nick can most simply generate a unidirectional 'rolling circle' and occurs in the replication of the double-stranded DNA bacteriophage ϕ X174.

The new DNA sequencing technology has already been used to determine the sequences at or near both types of origin, including those of phages ϕ X174 and λ , plasmid ColEI and the eukaryotic viruses SV40 and polyoma. F. Blattner's group in Madison has isolated and sequenced a functional λ origin (*Science* **198**, 1041; 1977). Their sequence contains a number of long pyrimidine tracts (one of 18 nucleotides), many runs of greater than four identical nucleotides and a number of direct, inverted and palindromic repeats that allow hairpin loops to be constructed. Unfortunately the functional significance of these features remains obscure, as does the nature of the initiation of λ replication. ColEI vegetative replication is initiated from a RNA primer and Tomizawa and coworkers (*Proc. natn. Acad. Sci. U.S.A.* **74**, 1865; 1977) have sequenced the region about the RNA/DNA junction, which is A-T rich and is preceded one to two helix

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turns before by a G-C rich region. Such a structure is reminiscent of known RNA termination sequences (*Nature* 271, 275; 1978). The same sort of distribution of A-T and G-C rich regions can also be seen in λ and φX174.

In Utrecht, Langeveld *et al.* (page 417 in this issue of *Nature*) have sequenced the region on the 3'OH side of the specific nick in duplex φX174 DNA at which replication is initiated. This nick is introduced by the *cis*-acting φX174 A protein. Reassuringly, this sequence is present in the φX174 total sequence (*Nature* 265, 687; 1977). Adjacent to the nick is a rotationally symmetrical octanucleotide which could be a specific (A-protein?) protein-binding site.

Initiation is likely to be controlled by replicon-specific 'signals' that allow or prevent replication. φX174 and λ each produce initiator proteins (A and O respectively) and it is perhaps not surprising that the genes for these proteins overlap the respective origins of replication. The *E. coli* chromosomal origin is also close to the *dnaA* gene which is involved in replication initiation. In contrast, since ColE1 is able to initiate replication in the absence of ColE1-specified proteins, its initiation may be controlled by a repressor.

The φX174 A protein appears to be the prototype of a range of important enzymes which nick DNA but conserve the phosphodiester bond energy by becoming covalently bound to the 5' end of the nicked strand (possibly through a phosphoamide bond, as in the ligase-AMP complex); they are subsequently able to religate the 5' end to the original or possibly a new 3'OH with little or no further energy input. With φX174 this allows a simple mechanism for the production of single strand circular viral strands by a modified 'rolling circle' model (Eisenberg *et al. Proc. natn. Acad. Sci. U.S.A.* 74, 3198; 1977). By analogy, a simple mechanism for conjugal transfer of plasmid DNA and its recircularisation

in the recipient presents itself. Not surprisingly then, ColE1 'relaxation complex', which has similar properties to the φX174 A protein/DNA complex, seems to be involved in conjugal transfer rather than vegetative replication, since work in our laboratory has shown that deletion of the specific site of ColE1 relaxation complex nicking abolishes conjugal transfer without noticeably affecting vegetative replication. It would seem that this site is a transfer origin. An SV40 'relaxation complex' that can be nicked close to the origin has also been described (Kasamatsu & Wu *Proc. natn. Acad. Sci. U.S.A.* 73, 1945; 1976).

DNA nicking-closing enzymes also belong to this class. A particularly interesting example is *E. coli* DNA gyrase, which *in vivo* introduces negative superhelical turns into covalently closed DNA. Nalidixic acid, a potent inhibitor

of DNA replication has recently been shown to interact with DNA gyrase. *In vitro*, gyrase can introduce negative superhelicity (in the presence of ATP), remove superhelical turns, and in the presence of nalidixic acid and SDS can cleave seemingly specific phosphodiester bonds, becoming covalently bound to the 5' end of the breaks in the process (Sugino *et al. Proc. natn. Acad. Sci. U.S.A.* 74, 4767; 1977; Gellert *et al. Proc. natn. Acad. Sci. U.S.A.* 74, 4772; 1977). Since introduction or removal of superhelical turns needs continuous nicking-closing activity, the conservation of phosphodiester bond energy is clearly important. DNA gyrase has been clearly implicated in DNA replication, yet its *in vitro* properties also make it, or similar 'break-rejoin' enzymes, candidates for involvement in recombination and transposition. □

A new type of chemisorptive bond

from R. H. Williams

DURING the past few years there has been great progress in our understanding of atomically clean solid surfaces and of the nature of chemisorption on them. This has been mainly due to the combination of new surface sensitive electron spectroscopic and other techniques (see Woodruff *Phys. Bull.* 27, 165; 1976) with sophisticated theoretical calculations of the surface on an atomic level. These studies are important in understanding processes such as catalysis, corrosion or the behaviour of many electronic devices.

Silicon has been particularly thoroughly studied in this respect because of its great technological importance and the availability of ma-

terial of high perfection. Clean (111), (110) or (100) silicon surfaces contain bonds which are not attached to other atoms, the so called 'dangling bonds'. For the (111) surface there is just one dangling bond per surface atom, extending out normal to the surface. Adsorbed gases such as chlorine bind to this bond, as illustrated in the beautiful recent angularly resolved photoelectron spectroscopic experiments of Larsen, Smith and Farrell (*Proc. V Int. Conf. Vacuum Ultraviolet Rad. Phys. Montpellier*, Extended Abstracts, II, 241; 1977).

On the theoretical front calculations of the surface electronic and vibrational structure have been made by several groups. In particular Appelbaum and Hamann at Bell Telephone Laboratories have conducted a series of

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calculations in which the charge and potential are treated self consistently (see *CRC Crit. Rev. Solid State Sci.*, August, 357; 1976) for a number of plausible clean surface structures and ones covered by adsorbed atoms. Although low energy electron diffraction gives the symmetry of the surface unit mesh the exact positions of the surface atoms are not known precisely. Figure 1a shows the form of the (100) surface, the atoms labelled 1 being the topmost layer, 2, 3, and so on being in subsequent layers. Atoms 1 have two unsaturated bonds. Obviously gases such as atomic hydrogen form strong covalent bonds with these unsaturated electrons. Since all the bonds associated with atom 2, in the second layer are saturated one would not expect hydrogen to form covalent bonds with these atoms.

In a recent paper, however, Appelbaum, Hamann and Tasso (*Phys. Rev. Lett.* **39**, 1487; 1977) conclude that if a hydrogen atom is located directly above atom 2 in Fig. 1 then it can form an unconventional type of covalent bond with the underlying silicon atom. As the hydrogen atom is brought to the surface immediately above atom 2 the charge distribution in the system as well as the force between the atoms is calculated as a function of separation d . It is shown that the hydrogen atoms have little effect on the dangling bond states. For $d = 3.7$ a.u. there is an attractive force between the hydrogen and silicon and as d decreases the attractive force also decreases until $d \sim 2$ a.u., with an accompanying polarisation of the charge around the silicon atoms in the second layer. It then increases rapidly until $d \sim 1$ a.u. and subsequently again decreases, reaching zero for $d \sim 0.25$ a.u. For values of d below 1 a.u. the behaviour of this force relationship is similar to that for hydrogen above a surface silicon atom. In addition the bond charge between the first and second layer silicon atoms is reduced by about 20%, weakening the bond and presumably allowing the bond distance to expand. In fact a simple interpretation of this unconventional covalent bond is that it is made up from valence-bond orbitals centred on the bond charge between the first and second layer Si atoms, and the hydrogen 1s state, somewhat analogous to the multicentred bonds formed in some boron compounds. The bonding combination leads to a tightly bound state, the anti-bonding combination yields a partially occupied state in the Si band gap, whilst the non-bonding combination weakens the Si-Si bonds. In addition the polarisation surface state noted for large d drops to an energy ~ 1.5 eV lower than for the conventionally bonded hydrogen on Si (111). It seems very likely that this

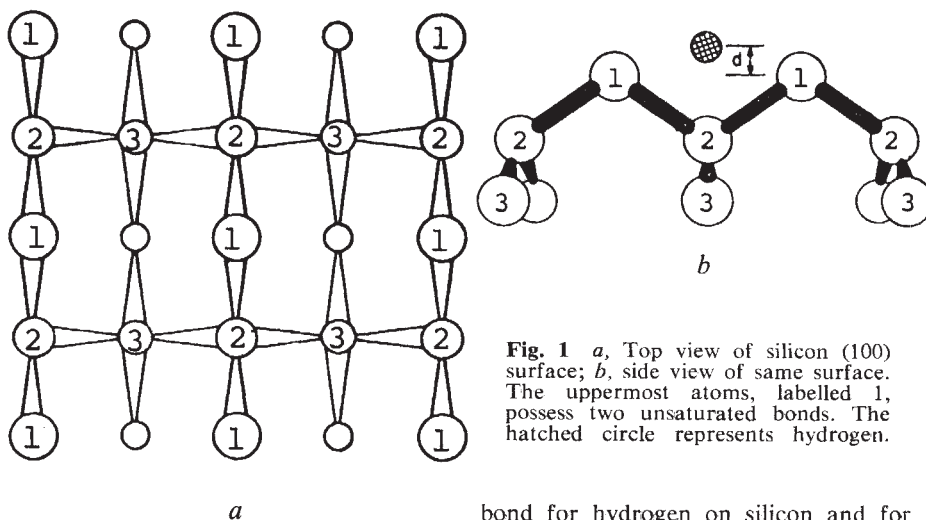


Fig. 1 a, Top view of silicon (100) surface; b, side view of same surface. The uppermost atoms, labelled 1, possess two unsaturated bonds. The hatched circle represents hydrogen.

state has been detected experimentally in the photoemission experiment of Sakurai and Hagstrum (*J. Vac. Sci. Tech.* **13**, 807; 1976) when hydrogen was adsorbed on Si (100) at room temperature.

Undoubtedly there will be further experimental and theoretical investigations of this new type of chemisorptive

bond for hydrogen on silicon and for other systems. It is of interest to note that such bonds may be of importance in amorphous silicon films prepared from silane by the R.F. glow discharge method and first prepared in n and p type form by Spear and LeComber (*Phil. Mag.* **33**, 935; 1976) at the University of Dundee. This material incorporates hydrogen in the lattice and promises to be of considerable use for manufacturing cheap solar cells. □

A new generation of elementary particles

from F. E. Close

EACH year the British high energy physics community meets at the Rutherford Laboratory to review the status of the field. In the past 3 years the discoveries of charm and the unification of two of the natural forces (electromagnetism and the weak force of radioactivity) have been highlights of the meetings. This year's meeting was perhaps even more exciting as discoveries in the past few months have heralded the opening of a new chapter in our conception of the microworld.

Three generations of elementary particles

Our everyday world can be loosely thought of as built from a hydrogen atom template (an electron and proton system) with neutrons (forming nuclear isotopes) and the radioactive phenomenon where a neutron transmutes into the proton with emission of an electron and also a ghostly neutrino (ν_e). The electron and neutrino are a pair of elementary particles called leptons. The proton and neutron on the other hand are now believed to be clusters of elementary particles called quarks. The quarks in the proton and neutron are of two varieties (or flavours), 'up' (u) and 'down' (d). Particles like the proton are made up of three quarks—two ups and one down for the proton itself—whereas

the other class of particles called mesons, that act as the carriers of the strong force between protons and neutrons, contain a quark and an antiquark. The best known meson is the pion (π) which is a combination of an up and a down quark but there are many other mesons, such as the heavier ρ meson and the much-disputed A_1 meson.

This pair of up and down quarks has many properties in common with the lepton pair and there appears to be a deep connection between them. These lepton and quark pairs are today known as the 'first generation' of elementary fermions.

For a reason not yet understood Nature repeated itself. The muon (seemingly a heavy version of the electron (see *News and Views* **266**, 679; 1977)) was discovered over a quarter of a century ago. It too is partnered by a neutrino and hence there is a 'second generation' of leptons (μ , ν_μ). We now know that there is also a second generation of quarks. The strange particles contain a strange (s) quark. The discovery of the J/ψ in 1974 was the first evidence for a charmed quark (c). In 1976 it was found that the c and s quarks are indeed brothers, dramatically in accord with theoretical predictions (*Nature* **262**; 537; 1976).

Charm, the sensation of the past 3 years, took a back seat this year. Interesting discoveries were reported which

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further support the charm hypothesis (see also *News and Views* 269, 286; 1977) and these are adding greatly to our understanding of quark dynamics. While much exciting work still lies ahead, charm is now 'in the textbooks', and the discovery of the decade was overshadowed this year by the astonishing discovery that Nature seems to employ a 'third generation' of elementary particles.

A new heavy lepton — τ

For 2 years there have been signs that a new heavy lepton τ exists. Although the consensus of opinion supported this view there was always some doubt as to whether the signals that were being attributed to the τ (whose mass was around 1,800–1,900 MeV) were really arising from decays of the lightest charmed particles (1,865 MeV).

A very important result seems to be emerging from DORIS, the e^-e^+ colliding beam facility at Hamburg. Since the lightest charmed particle is at 1,865 MeV then 3,730 MeV of energy is needed to produce pairs of charmed particles. Hence the particle ψ' (3,684 MeV) cannot decay into pairs of charmed particles and yet the signals that seem to characterise the production of pairs of the hypothetical τ lepton are being seen in decays of ψ' (3,684). Hence the signals are genuinely from τ , and not charm. Moreover the mass of τ is now believed to be within 20 MeV of 1,810 MeV, some 50 MeV lighter than the lowest charm mass.

Decay properties have been studied and are in line with what would be expected if the τ is accompanied by a new neutrino ν_τ (analogous to the ν_e and ν_μ first and second generations). There is however a tantalising puzzle: the τ is seen to produce the ρ in its decays in accord with calculation; also the A_1 is seen (confirmation of the A_1 is an interesting 'old' physics by-product of this new world); however, no sign of the π is seen. This should be produced in $\tau \rightarrow \pi + \nu_\tau$. One suggestion put forward is that the ν_τ has spin 3/2 (unlike spin 1/2 for ν_e , ν_μ). If so, a whole new ball game lies ahead.

Another possibility is that the experimentalists should look harder.

A fifth quark?

If there is a third generation of leptons, theorists have argued that there should be a third generation of quarks. This new quark pair is labelled t and b for 'top' and 'bottom'.

In the summer of 1977 a massive Y (upsilon) particle (mass 9.4 GeV—three times that of the ψ , and nine times heavier than the proton) was seen in muon pairs produced by proton-proton collisions at Fermilab. It is now confirmed that there is also an Y' some 600 MeV heavier than Y . This is tantalisingly the same mass differences as in $\psi' - \psi$ and so theorists speculate that these Y are bound states of a new quark (bottom seems to fit

better with the production rates than does top), and that the quarks feel a potential that rises logarithmically with the inter-quark separation (since this leads to splittings in mass for $\psi'\psi$ or $Y'Y$ independent of the mass scale).

Confirmation of the Y world has come from the intersecting storage ring at CERN where an enhanced production of lepton pairs is seen in the 9–10 GeV region—their resolution cannot separate Y and Y' however.

Bottomless particles?

The ψ had hidden charm—formed by a charmed quark and a charmed antiquark binding together producing a particle with net charm zero—and particles with naked charm were subsequently found. Hence, if the Y has 'hidden bottom' (made from bottom quark and its antiquark) then one predicts that 'naked bottom' particles will exist. Similarly if top quarks exist then 'naked top'—or 'topless' states will eventually be found. The prudish may care to note that t and b are said to stand for truth and beauty, rather than top and bottom, by some physicists. It is predicted that these will decay in a cascade as follows: top decays weakly to bottom, bottom to charm and charm to strange. At each stage a lepton can be emitted. Hence one might anticipate that when a neutrino interacts with a proton several leptons may be produced, for example

$$\nu + p \rightarrow \begin{matrix} \text{"t..."} \\ \swarrow \quad \searrow \\ \mu^- \quad \mu^- \\ \quad \quad \quad \swarrow \quad \searrow \\ \quad \quad \quad b\mu^+ \quad c\mu^- \\ \quad \quad \quad \quad \quad \quad \searrow \\ \quad \quad \quad \quad \quad \quad s\mu^+ \end{matrix}$$

A beautiful event with four energetic muons was reported at CERN last month. It is still too soon to say whether this is the first clue in the search for topless and bottomless particles or whether it is a manifestation of something 'conventional' like charm.

Where now?

The phenomenology of bottom quarks was described by J. Ellis (CERN). In particular he drew attention to work of Kobayashi and Maskawa in Japan (in 1972) which showed that the mysterious phenomenon of CP violation emerges rather naturally in a world with six quarks. If the third generation of quarks is indeed filled by subsequent discovery of the top quark then this phenomenon may at last be understood. The search for 'top' is now on.

H. Fritsch (CERN) led a discussion of the impact of the new discoveries on attempts to build gauge field theories unifying weak, electromagnetic and strong interactions. The latter part of this idea is exploitation of the theory of quantum chromodynamics (QCD) which has been developed over the past few years as a quantum field theory of strong interactions which describes the interactions

between quarks. G. Parisi (Ecole Normale, Paris) described some of the phenomena that QCD predicts should be seen in highly inelastic lepton scattering on protons and nuclei. T. Quirk (University of Oxford) showed data from such experiments which appear to be in accord with QCD predictions.

H. Lipkin (Weizmann Institute, Israel) described some exotic multiquark states that should exist even with the first generation of quarks (see *News and Views* 266, 201; 1977) and discussed evidence for 'exotic nuclei' (dibaryon resonances) that may fit in with these ideas.

The evidence that Nature exploits gauge theories has led to an upsurge of interest in them and E. Corrigan (University of Durham) described some of the rich and exciting structure that is emerging from these studies. Theorists are preparing themselves for the possibility that we are now *en route* for a grand synthesis of all natural forces, including gravity. G. Gibbons (University of Cambridge) described recent work in quantum gravity. Physicists already punch drunk with new quarks, new leptons, monopoles and merons, were now introduced to gravitational monopoles, nut and bolt solutions and even had to contemplate a foam of black holes. When all of these incredible discoveries are finally welded together a profound conception of Nature seems likely to emerge that will make Star Wars seem child's play. □

Homo or hetero?

from Robert W. Cahn

At first sight, an ordered metallic solid solution is as simple and perfect as a crystal can be. Solvent atoms sit on one kind of lattice site, solute atoms on another. The supposed simplicity and perfection are deceptive, for any ordered alloy differs from a plain inter-metallic compound in that its ordered structure is unstable and dissipates on heating before the solid melts. The interest of such alloys does not lie in the perfection of the order, but in the mysterious intermediate states in which order begins to disappear; the halfway house between perfect crystallographic order and an utterly random distribution of atoms on the available lattice site. Are the wrongly sited atoms scattered at random among the 'right' ones—is there a randomness within the randomness—or are there minute groups of 'wrong' atoms, right with respect to their immediate neighbours but wrong with respect to the main mass of the ordered crystal? The first is a homo-

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geneous model of partial order, the second, a heterogeneous one. The homo/hetero dispute goes deep in this field of solid state physics.

When an ordered alloy is heated above its critical temperature, all long-range order disappears, but local or short-range order persists. Its persistence may be deduced from the observation of diffuse X-ray scattering in place of sharp superlattice lines, or from small thermal, electrical or dilatometric anomalies. The atomic format of short-range order is subject to the same uncertainty between homogeneous or heterogeneous models. One view is that a short-range-ordered alloy is homogeneous in the sense that any solvent atom has statistically the same likelihood of having like or unlike nearest neighbours as any other solvent atom. Of the several heterogeneous models, the most intriguing postulates a dispersion of tiny fully ordered domains surrounded by a wholly random matrix with a thin boundary in which there is a gradient of the degree of order. In other words, ordered 'precipitates' exist in a disordered matrix. This model, associated with H. P. Aubauer, has occasioned a great deal of controversy since it was first advanced 6 years ago. The difficulty is this: in the normal way, any dispersion of precipitates in a matrix is unstable; the Matthew Principle operates and large precipitates grow at the expense of the small. The same happens with soap bubbles or with raindrops in a cloud. Aubauer (*Acta Met.* **20**, 165, 173; 1972) pointed out that a short-range-ordered, heterogeneous alloy remains indefinitely in a state of fine dispersion: the ordered microdomains do not grow however long they are heated. Aubauer attributes this to the presence of an elastic strain gradient around each domain (because the volume per atom is different in ordered and random regions), and the elastic energy is shown to inhibit the coarsening of domains. Aubauer's calculations have repeatedly been challenged on thermodynamic grounds (the story of the dispute is neatly anatomised by J. W. Martin and R. D. Doherty on pages 205-209 of their book, *Stability of Microstructure in Metallic Systems* (Cambridge University Press, 1976).

The homogeneous/heterogeneous dichotomy with regard to short-range order seems to be incapable of final resolution by X-ray diffraction, electron diffraction, electron microscopy or electrical resistivity. Copper-rich, alpha-phase Cu/Al solid solutions in particular have been examined by all these techniques, over a period of 20 years, and though the partisans of heterogeneity have the edge, the arguments have been impossible to resolve decisively.

A deceptively simple investigation has now, to all appearances, tilted the balance firmly in favour of a heterogeneous model (although that still leaves open the question whether Aubauer's particular version is the most apposite). L. Trieb and G. Veith (*Acta Met.* **26**, 185; 1978), two Viennese physicists working in consultation with Aubauer, have studied several Cu/Al alloys exclusively by measurements of electrical resistivity. In the past (for instance in a classic study by Wechsler and Kernohan, *Acta Met.* **7**, 599; 1959) this has been done by quenching from high temperatures, which leads to great complexities arising from quenched-in vacancy populations which lead to kinetic anomalies. Trieb and Veith avoided large temperature changes: an ultraprecise measurement technique allowed them to cool or heat their samples by only 5 °C, so that in effect the only variable to affect the resistivity was a change in degree of short-range order. By approaching the same end-state from above and below, they were able to define the temperature range in which equilibrium was attainable in reasonable times in their various alloys. They then analysed mathematically the form of the kinetic curves and showed unambiguously that these could only be interpreted on the hypothesis that two different processes were in progress at different rates. Twin-process kinetics cannot be reconciled with a homogeneous end-structure. Trieb and Veith point out that in Aubauer's 'disperse order' model, an alloy of given composition has at each temperature, in equilibrium, a defined volume fraction of ordered domains and a defined mean size of domains. They propose that the equilibrium volume fraction is attained several times faster than is the equilibrium degree of dispersion, following any sudden small change of temperature. The electrical resistivity is sensitive to both variables and can thus be used to follow their changes. For the most concentrated alloy (18 at. % Cu) the disordered matrix virtually disappears and is replaced by antiphase boundaries.

Trieb and Veith have made a very convincing case for the correctness of a heterogeneous model and have shown how powerful a basically very simple technique, allied with extreme precision and careful quantitative analysis, can be.

It is intriguing that the homo/hetero dispute extends also to the structure of oxide glasses. An enormous amount of work has gone into analysis of such glasses by diffuse X-ray scattering, but it is fair to say that the resultant pair-distribution functions cannot reliably discriminate between homogeneous (Zachariasen-type) network models and various models based on non-crystalline

clusters, or clusters of microcrystals of two polymorphic variants in co-existence. This battleground of glass structures still awaits its resolution, which will perhaps come, in due course, from some technique as elegant and simple as that applied by Trieb and Veith to short-range order.

Their study has shown again the limitations of microscopy, however great the resolution and however sophisticated the technique, in resolving details of not-quite-perfect structures. □

Negative deflection angles in heavy ion scattering

from P. E. Hodgson

WHEN two nuclei interact at energies high enough to overcome their mutual electrostatic repulsion their quantum-mechanical wavelengths are usually small compared with their own radii, so that it is possible for many purposes to consider them as classical particles moving along well-defined orbits. If we then think of the scattering angle as a function of the impact parameter we find several curious effects, in particular that several impact parameters can give the same scattering angle. It has now proved possible to distinguish these orbits experimentally.

The orbits can be visualised by considering the interaction for various impact parameters, or distances of closest approach in the absence of all interactions. For large impact parameters the projectile feels only the long-range repulsive Coulomb (or electrostatic) field and is deflected through a small positive angle. As the impact parameter is decreased the nuclei come closer together so the Coulomb interaction is larger and the repulsion and thus the deflection angle is greater. Thus initially the deflection angle increases as the impact parameter decreases.

As the impact parameter is still further decreased the nuclei approach close enough for their short-range attractive nuclear fields to interact. These oppose the repulsive Coulomb force and thus reduce the scattering angle. Eventually as the impact parameter is reduced still further there comes a point when the Coulomb and nuclear fields exactly balance and the particle is not scattered at all. At still smaller impact parameters the scattering angle becomes negative, and the corresponding orbits wind round the

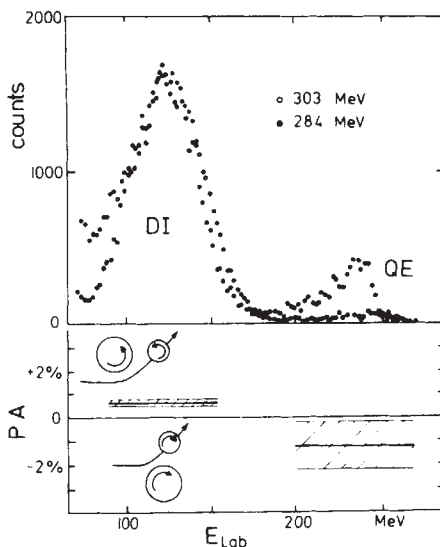
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back of the target nucleus as shown in the inset to the figure.

Although there is a clear physical difference between the orbits corresponding to positive and negative scattering angles, they cannot be distinguished experimentally just by studying the differential cross sections, since equal number of particles are incident on both sides of the target nuclei. Thus until recently it was not possible to confirm these negative deflection angles experimentally, although they are theoretically very plausible.

It has now been shown by Trautmann and colleagues (*Phys. Rev. Lett.* **39**, 1062; 1977) that it is possible to do this by measuring the polarisation of the residual nuclei, which depends on whether the scattering was to positive or negative angles. This is because in these interactions some of the orbital angular momentum of the projectile is given to the target nucleus, so that it is left spinning. If now we consider two orbits that both give emergent particles in the same direction, but one corresponding to positive and the other to negative scattering angle, we find that they leave the residual nucleus spinning in opposite directions, as shown in the inset to the figure. This may easily be seen physically by using the concept of tangential friction; the interactions that take place as the nuclei brush past each other transfer energy and this is felt by the projectile as a tangential force tending to set it into rotation; the same force also sets the target nucleus into rotation.

The particles that have traversed the two types of orbit can be distinguished by the amount of energy they have lost in the interaction. Those scattered through positive deflection angles have been strongly repelled by the Coulomb field and weakly attracted by the nuclear field. Since the loss of energy and the transfer of particles takes place mainly through the nuclear field, such particles will on the average lose rather little energy. On the other hand those particles that are scattered through negative deflection angles have been acted on by a relatively weak Coulomb field but a very strong nuclear field, and so will have lost on the average much more energy. This is illustrated by the figure, which shows the energy spectrum of the particles emitted at 35° from the interaction of 284 and 303 MeV, argon nuclei with a target of natural silver. There are two distinct peaks, the one at higher energies corresponding to the particles that have lost rather little energy in quasi-elastic interactions, and the one at lower energies that have lost much more energy in deep inelastic inter-



The upper half of this figure shows the coincident energy spectra of emerging particles with charges between 11 and 21 from the interaction of 284 and 303 MeV argon nuclei with natural silver. The deep inelastic and quasi-elastic groups are clearly distinguished. The lower part of the figure shows the corresponding polarisations, and the inset shows how these follow from the classical orbit picture of the interaction.

actions.

It is possible therefore to identify the particles that have been scattered through positive and negative deflection angles so all that remains is to show that they have indeed left the residual nuclei spinning in opposite directions. This was done by measuring the circular polarisation of the de-excitation gamma rays corresponding to the two groups. In the initial stages of the de-excitation process charged and neutral particles are emitted, and this will in general reduce the value but not change the sign of the nuclear polarisation. Thus the circular polarisation of the gamma rays emitted in the last stages of the de-excitation process, which has the same sign as the nuclear polarisation, tells us about the direction of spin of the residual nucleus immediately after the interaction.

The results of the measurements of the circular polarisations of the gamma rays in coincidence with the two groups of particles are shown in the figure. Those corresponding to the negative deflection angles have a small positive polarisation, whereas those corresponding to positive deflection angles have a small negative polarisation, which is just what we expect from the theory of the process. This agreement with the theory of the orbits responsible for the positive and negative scattering angles and the energy losses of the particles in the two groups confirms the overall correctness of the picture. This is a remarkable illustration of the value of

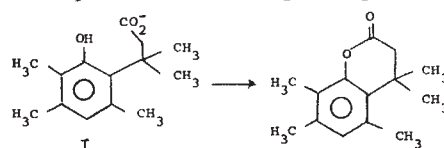
classical arguments in understanding the details of interactions between nuclei at high energies. □

What's special about enzymes?

from G. C. K. Roberts

A Ciba Foundation Symposium on Molecular Interactions and Activity in Proteins was held at the Ciba Foundation on 6-7 December 1977. It was organised by Professor A. R. Battersby, University of Cambridge. The Proceedings will be published by Elsevier/Excerpta Medica/North Holland.

THE symposium was devoted to a discussion of the special catalytic powers of enzymes, and the extent to which this can be explained in precise chemical terms. W. N. Lipscomb (Harvard University) began by summarising the ways in which the remarkable rate enhancements observed with enzymes might be achieved, emphasising that enzymes operate by normal chemical mechanisms, using specific binding to speed things up. He went on to describe model systems which illustrate some of the contributions to enzymic catalysis. Perhaps the most striking example was



the reaction which, due to the 'locked' conformation of compound I is 5×10^{10} times faster than the reaction of the corresponding compound without the methyl groups (Milstein & Cohen *J. Am. Chem. Soc.* **94**, 9158; 1972).

It was clear from the examples shown that there are enough factors demonstrable in model systems to account for the power of enzyme catalysis. In which case, what about building a 'model enzyme'? The early stages of an attempt in this direction were reported by J. R. Knowles (Harvard University), who described the design and synthesis of a modified cyclodextrin as a catalyst of phosphate ester hydrolysis. Aromatic molecules are known to bind in the central 'hole' of cyclodextrins, while the catalytic site was provided by three $-NH_3^+$ groups positioned so as to stabilise the transition state. Unfortunately, testing of this ingenious compound as a catalyst was not complete at the time of the meeting; however

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specific binding was demonstrated and it promises to give considerable insight into the requirements for optimal catalysis. Another area in which the organic chemical approach has made enormous contributions to our understanding of enzymes is that of stereochemistry; D. Arigoni (ETH, Zurich) described an elegant series of studies of biological methylation reactions using S-adenosyl methionine labelled with a chiral methyl group (—CHDT).

F. M. Richards (Yale University) described computations of the solvent-accessible surface area of proteins; contrary to earlier generalisations, the surface of a protein is made up of polar and non-polar groups in roughly equal proportions. Richards showed that calculations of changes in surface area can be used to estimate 'hydrophobic' interactions, and that they could form the basis of a program which searched for optimal helix-helix contacts, and which worked well for myoglobin—an encouraging step towards an algorithm for protein folding. He also pointed out that calculations of the packing density of atoms in a protein lead to a 'solid-like' picture of the interior of a protein. This raised the question of flexibility in proteins which was taken up by a number of other speakers throughout the meeting. D. M. Blow (Imperial College, London) discussed crystallographic evidence for large scale flexibility in some proteins, particularly Huber's work on trypsinogen and his own on tyrosyl tRNA synthetase, in which about a third of the subunit fails to give rise to any interpretable electron density. K. Wüthrich (ETH, Zürich) and R. J. P. Williams (University of Oxford) drew attention to NMR evidence for mobility in proteins, principally at the level of single residues. It seems clear that proteins in general are more flexible than the 'solid-like' description implies, although the degree of flexibility varies substantially, not only from one protein to another but within one protein—as exemplified by pancreatic trypsin inhibitor. As pointed out by Wüthrich, this protein is both rigid, in that several peptide NH protons fail to exchange with the solvent on a timescale of years, and flexible, in that the aromatic rings of its tyrosine residues are able to 'flip' through 180° at rates of the order of 10^3 s^{-1} . The possibility that flexibility is in some way essential to catalysis was discussed both by Williams and also by B. Vallee (Harvard University) in describing his studies of arsanilazo-tyrosine 248 carboxypeptidase. Optical absorption, circular dichroism and resonance Raman spectroscopy were used to obtain structural information on the environment of the 'probe', which

could then be related to individual kinetic steps in catalysis.

Turning to substrate binding, C-I. Brändén (Uppsala) described crystallographic work on liver alcohol dehydrogenase, including studies of a ternary complex, enzyme.NAD⁺.p-bromobenzyl alcohol. In this enzyme, the substrate binds at the bottom of a very deep pocket, lined exclusively with hydrophobic residues, where it coordinates to the zinc atom—the only specific enzyme-substrate interaction that is apparent, thus explaining the very broad specificity of the enzyme. Brändén reported that it is very difficult to say from the crystal structures why substrates bind 2–3 orders of magnitude more tightly in the presence of coenzyme; a subtle change in the electronic structure of the zinc may be involved. H. Gutfreund (University of Bristol) described transient-state kinetic studies of the alcohol dehydrogenase reaction, presenting evidence for proton release accompanying the binding of NAD⁺. This proton may well originate from a 'charge relay' system, comprising Asp 293–His 151–Ser 48–(water or alcohol)–zinc, at the active site. An interesting, though as yet unexplained, generalisation noted by Brändén is that in almost all enzymes having a parallel β -sheet structure, the C-terminal part of the sheet contains an anion binding site; in dehydrogenases the pyrophosphate of the coenzyme binds here, in carboxypeptidase the carboxylate of the substrate, and in rhodanese a thiocyanate ion.

Several other speakers took up the question of the specificity of binding in a pharmacological context; G. C. K. Roberts (National Institute for Medical Research, London) discussed the origins of the very tight binding of inhibitors to dihydrofolate reductase, emphasising the role of conformational changes, while M. Halsey (Clinical Research Centre, London) described studies of the binding of general anaesthetics to haemoglobin. T. Blundell (Birkbeck College, London) showed how the combination of the crystal structure with studies of modified insulins makes it possible to define those regions of the molecule which most probably interact with the receptor.

The reactivities and pK_a values of side chains on enzymes can be markedly altered from those of the free amino acids, and this can make a significant contribution to the observed rate enhancement. D. C. Phillips (University of Oxford) and Williams described a combined onslaught, by crystallography and NMR, on lysozyme, asking whether one could predict the reactivity of pK_a of a side chain from its environment as revealed by crystallography. While the exchange rate of the indole NH protons of the

tryptophan residues, for example, correlated well with their solvent accessibility, this was not the case for iodination of the tyrosine residues—the most readily iodinated tyrosine was neither the most solvent accessible nor the one with the most 'normal' pK_a .

Perhaps the most remarkable example of a special environment for a side chain of an enzyme was described by B-M. Sjöberg (Stockholm). Ribonucleotide reductase contains a stable free radical, which is most probably involved in the activity of the enzyme, and which is formed on the side chain of a tyrosine residue, the spin density being located on carbons 1, 3 and 5 of the aromatic ring. The stability of such a radical during purification and handling of the protein is really quite extraordinary.

In his summary, Lipscomb noted that, though we are fairly sure we understand the general principles governing the behaviour of enzymes, we do not know exactly how things work in any individual case. This clearly applies not only to catalytic activity but also to the details of specificity and reactivity. The fact that precise chemical questions can be asked about such systems, if not yet answered, is a mark of the progress that has been made, but theoretical advances will be required before we can properly relate structure and energetics. □



A hundred years ago

ON Monday afternoon a powerful shock of earthquake was felt in the island of Jersey. It was so strong as to cause houses to totter and bells to ring. Its course was from east to west. There was at the time a heavy gale from the south-west in the English Channel. At 11.55 A.M. the same day a shock, lasting about four seconds, was felt at Eastern Alderney. No doubt it was the same earthquake which was felt at Brighton, Blackheath, Fareham, and St. Leonards, as reported in yesterday's *Times*, and at Paris, Havre, and Rouen, as stated by the *Times* Paris correspondent. Mr. Dobson, writing to us from the Royal Victoria Hospital, Netley, Southampton, states that the first shock occurred there at seven minutes to twelve o'clock exactly, and lasted about five or six seconds. It was sufficiently strong to cause the door to shake with some violence, and many objects in the room continued to vibrate for a considerable time.

From *Nature* 17, 2 February, 272; 1878.

review article

Quantification of Earthquakes

Hiroo Kanamori*

Because earthquakes are the result of complex geophysical processes, it is not a simple matter to find a single measure of the size of an earthquake. Further, extremely large earthquakes saturate the conventional magnitude scale. Recent estimates of energy released by such earthquakes suggest values high enough to have possible implications for plate motion, the Chandler wobble and rotation of the Earth.

DURING 1976 a large number of destructive earthquakes occurred: on 4 February, Guatemala suffered an earthquake of magnitude 7.5, with the loss of 23,000 lives; on 6 May, an earthquake of magnitude 6.5 cost Italy 1,000 lives; on 27 July, an earthquake of magnitude 8.0 struck China with the loss of 650,000 lives. The Chinese (Tangshan) earthquake is one of the most disastrous events since the 1556 Chinese earthquake in which 830,000 people perished. Moreover, there have been several other destructive earthquakes in the same year: Fig. 1 clearly demonstrates that 1976 was one of the worst years for earthquake casualties in recent times. However, the damage caused by an earthquake depends not only on its physical size but also on various factors such as when and where it occurs, and Fig. 1 therefore does not necessarily represent the physical 'size' of earthquakes. The question of how we measure the 'size' of an earthquake has been historically a very important yet difficult seismological problem.

Earthquake magnitude scales and seismic energy

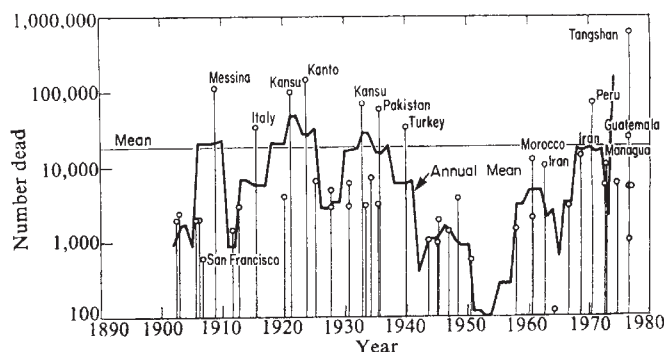
Since the physical process underlying an earthquake is very complex, we cannot express every detail of an earthquake by a single parameter. However, it would be convenient if we could find a single number that represents the overall physical size of an earthquake. This was the very concept of the earthquake magnitude scale, the so-called Richter scale, introduced by Charles F. Richter¹ in 1935. Richter used the maximum amplitude of seismic waves recorded by the Wood-Anderson seismograph. After correcting for the decay of the amplitude with distance from the epicenter, he established the local magnitude scale (M_L) for earthquakes in Southern California, using the logarithm of the observed amplitude of seismic waves. The seismic waves used for local magnitude have periods ranging approximately from 0.1 to 2 s, equivalent to a wavelength of 300 m to 6 km. Since Richter's scale was introduced more or less empirically, it was not quite clear in the beginning what physical parameter of an earthquake this scale represented. However, the Richter scale turned out to be extremely useful for various purposes. The method was so simple that magnitudes could be assigned to hundreds of earthquakes on a routine basis. Since M_L is defined in the period range where the effect of seismic waves on buildings is most pronounced, it is a very good measure of the strength of ground shaking at a given distance, and is very useful for various engineering purposes.

Because of this remarkable success of the M_L scale, Gutenberg² extended this kind of measure to earthquakes in distant sites.

For earthquakes at very large distances, seismic surface waves with a period around 20 s are often dominant on seismograms. Gutenberg therefore defined another magnitude scale, M_S , called the surface-wave magnitude, using the amplitude of surface waves with a period of 20 s. The wavelength of these surface waves is about 60 km. Gutenberg³ also used seismic body waves (P and S waves) to define another scale, m_b , called the body wave magnitude. The period of these body waves is usually from 1 to 10 s. Thus M_S and m_b represent different parts of the frequency spectrum of seismic waves, so that each of these scales represents a different physical parameter of an earthquake. However, extensive studies of Gutenberg and Richter suggested that m_b and M_S seem to be related to each other and could be used to represent a fundamental physical parameter, the energy of seismic waves, E . Through repeated revisions, Gutenberg and Richter⁴ finally derived a relation $\log_{10} E = 1.5 M_S + 11.8$ (E in ergs). This relation made it possible to estimate seismic energy from the magnitude M_S , which can easily be routinely measured. Of course, we cannot expect a very accurate estimate of seismic energy from such a crude parameter through such a simple relation. Nevertheless, this relation provided the only means for quantifying earthquakes in terms of the energy of elastic waves.

The monumental book of Gutenberg and Richter⁵, *Seismicity of the Earth*, catalogues most large earthquakes that occurred from 1904 to 1949. In the later work of Gutenberg⁶ and in the second edition of *Seismicity of the Earth* the catalogue was extended to the period from 1897 to 1952. Fortunately, after these catalogues were completed, a number of very large earthquakes occurred starting with the Kamchatka earthquake,

Fig. 1 Loss of life caused by major earthquakes. The vertical bars are for the individual event and the solid curve shows the annual average (unlagged 5-year running average).



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$M_s = 8\frac{1}{4}$ on 4 November 1952. Here, 'large' refers to the length of the fault. The 1952 Kamchatka earthquake was followed by the Fox (Aleutian) Island earthquake in 1957, ($M_s = 8\frac{1}{4}$), the Chilean earthquake in 1960 ($M_s = 8.3$), the Kurile Island earthquake in 1963 ($M_s = 8.1$), the Alaskan earthquake of 1964 ($M_s = 8.4$) and the Rat Island earthquake of 1965 ($M_s = 7\frac{1}{2}$). The faults which broke in these earthquakes are extremely long (as long as 800 to 1,000 km for the 1960 Chilean earthquake and the 1957 Fox Island earthquake). No faults that long are known to have ruptured for events before 1952, although the data before 1904 are incomplete. We will use the term 'great earthquakes' for these events with a very long fault.

Seismic waves generated by these great earthquakes are quite spectacular. Very long-period (200–300 s or even longer period) waves which last for several days after the earthquake are often recorded on long-period seismograms. However, the amplitude of relatively short-period (about 20 s) waves generated by these earthquakes is not particularly large, and is about the same as for 'ordinary' large earthquakes. Therefore M_s for these events is not particularly large, in spite of the extremely large fault length and the pronounced long-period waves. Since the energy was calculated only from M_s , the energy release of these 'great' earthquakes was thought to be comparable to that for other 'ordinary' large earthquakes. Why does the amplitude of short-period waves not grow as the length of the fault increases? The amplitude of seismic waves represents the energy released from a volume of crustal rock whose representative dimension is comparable to the wavelength. Several studies⁷ suggest that the energy density per unit volume of crustal rock is almost constant regardless of the size of the earthquake. Since the wavelength of seismic waves used for the determination of M_s is only about 60 km, the amplitude of 20-s waves does not increase as the fault length increases beyond 60 km; therefore the M_s scale saturates. Since these giant earthquakes occurred after the publication of *Seismicity of the Earth*, Gutenberg and Richter did not have the opportunity to consider the saturation problem.

Not much further effort was devoted to the question of energy estimation until the early 1960s, when remarkable progress was made in long-period seismology. Owing to the worldwide deployment of the standardised long-period seismographs and the development of the theory of the generation and propagation of very long-period (up to 1 h) waves, it became feasible to use very long-period waves for quantitative analyses of an earthquake source. Such long-period waves have wavelengths of several hundreds to several thousands of kilometers, which are comparable to the fault length of great earthquakes, and are therefore suitable for measuring the overall 'size' of great earthquakes.

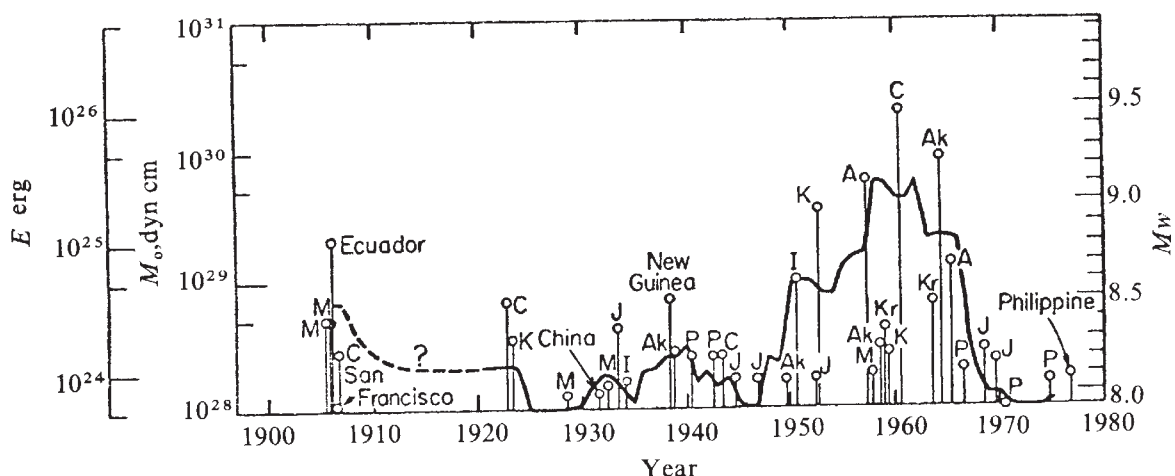
Seismic moment

One of the major breakthroughs in this field was made by the introduction of the concept of seismic moment. Aki⁸ applied elastic dislocation theory to the study of earthquake mechanism. According to elastic dislocation theory, the amplitude of very long-period waves is proportional to the quantity $M_0 = \mu DS$ where S is the surface area of the fault, D is the average displacement discontinuity on the fault plane and μ is the rigidity of the material surrounding the fault. M_0 is called the seismic moment. In order to obtain M_0 from the observed seismograms, the effect of propagation, attenuation and the geometry of the fault must be removed. Fortunately, unlike short-period (for example, 20-s) waves, these long-period waves are very coherent so that accurate measurements of M_0 are possible. Thus the seismic moment is one of the most reliable seismological parameters that represents the 'size' of an earthquake. Because M_0 does not saturate, it is a more suitable parameter to represent the size of great earthquakes than the conventional magnitude scales such as M_s .

Several attempts were made to use long-period (100–200 s) waves to quantify large earthquakes^{9–11}. Bruce and Engen¹⁰ introduced a new scale, 100 s magnitude M_{100} , which is based on the amplitude of surface waves with a period of 100 s instead of 20 s. This scale clearly demonstrated that long-period waves are very useful for distinguishing great earthquakes from ordinary large earthquakes.

In recent years, extensive efforts have been made to determine M_0 for great earthquakes as well as large and small earthquakes. The determination of M_0 for recent events can be made relatively easily and very accurately by using high-quality standardised seismograms. The determination of M_0 for old events is less accurate because of the poor quality of old seismograms. Nevertheless, by combining various techniques, the seismic moments of 44 earthquakes out of 52 events of $M_s \geq 8$ which occurred since 1921 have been estimated. For the period from 1904 to 1920, the data are not very complete. Fig. 2 shows the moments for the individual earthquakes as well as the annual average¹². Since the seismic moment directly represents the overall deformation at the earthquake source, Fig. 2 indicates the temporal variation of the amount of crustal deformation associated with earthquakes. It is clear that the seismic activity measured by the seismic moment was very high during the period from 1950 to 1965. The annual average seismic energy release during this period is almost two orders of magnitude larger than that during the period prior to 1950. Is this temporal variation real, or is it an artefact of the poor quality of the record for the period before 1950? Although the quality of the moment determination is somewhat poor before 1950, it is almost certain

Fig. 2 The seismic moment M_0 , seismic energy E , and the magnitude M_w of great and large earthquakes. The solid curve shows the annual average of seismic energy obtained by taking unlagged 5-year running average. A: Aleutian; Ak: Alaska; C: Chile; I: India; J: Japan; K: Kamchatka; Kr: Kurile; M: Mongolia; P: Peru.



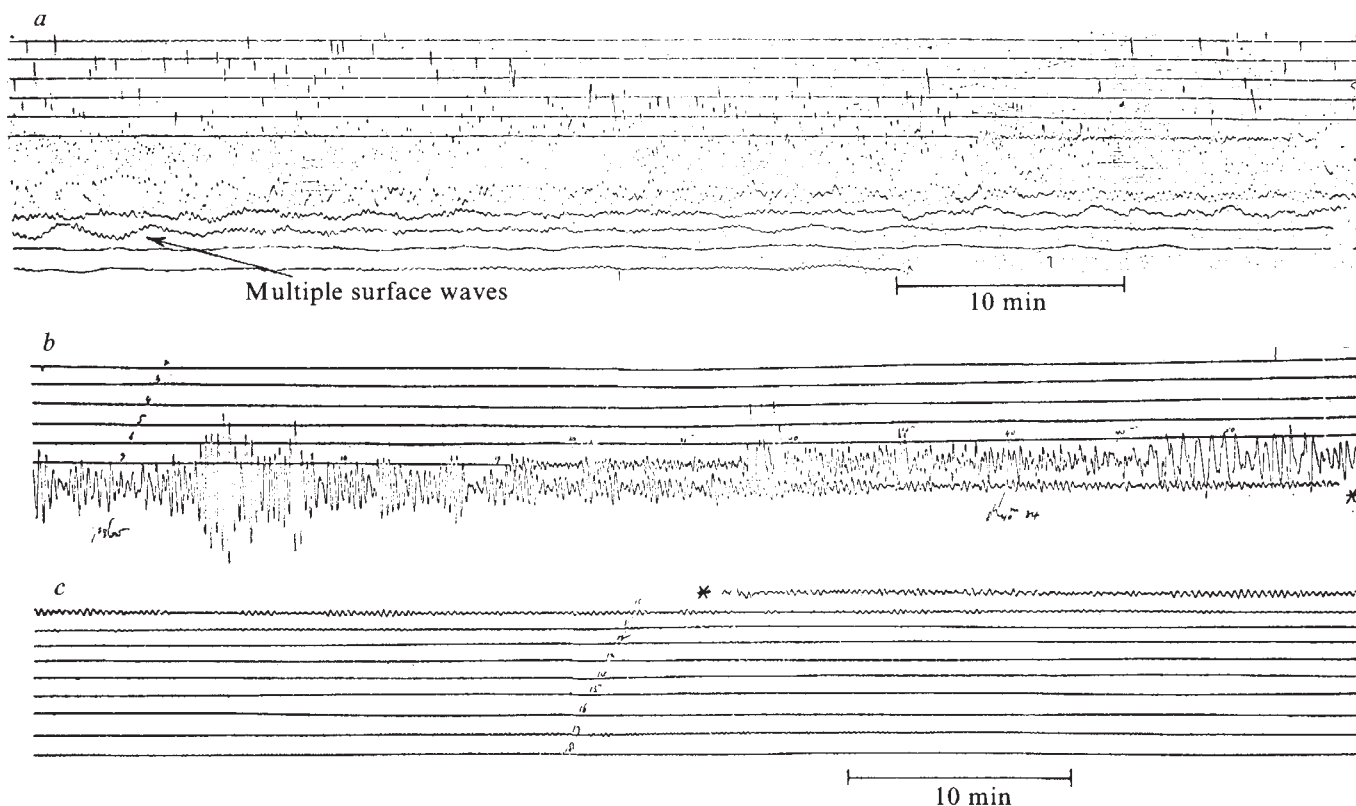


Fig. 3 Comparison of *a*, the 1960 Chilean earthquake ($M_s = 8.3$) and *b*, the 1929 Aleutian Island (Fox Island) earthquake ($M_s = 8.1$) recorded by the Milne-Shaw seismograph. The long-period surface waves—marked as multiple surface waves—which are clearly recorded for the 1960 Chilean earthquake distinguish a great earthquake (Chilean earthquake) from a large earthquake (Fox Island earthquake).

that no earthquake from 1920 to 1950 was as large as the 1960 Chilean earthquake. As shown by Fig. 3, the difference between 'great' and 'large' earthquakes is quite obvious even on old seismograms. Thus, the pattern shown in Fig. 2 is probably real despite the differing quality of the data.

It is also clear in Fig. 2 that the annual average has decreased very dramatically in recent years. This trend is opposite to the trend shown in Fig. 1. This negative correlation clearly suggests that the physical size and the social impact of an earthquake are not directly related.

Seismic moment and seismic energy

The seismic moment can be related to the energy released in earthquakes. Suppose the stress on the fault plane is reduced from σ_0 to σ_1 in an earthquake. Then the reduction in the strain energy is $\Delta W = \sigma D S$ where $\sigma = (\sigma_0 + \sigma_1)/2$ is the average stress, D is the average offset on the fault plane, and S is the area of the fault plane. Combining this with the expression for the seismic moment M_0 we have $\Delta W = (\sigma/\mu)M_0$. If we assume that the fault motion stops when the stress on the fault becomes approximately equal to the frictional stress σ_f , that is $\sigma_1 = \sigma_f$, the above equation reduces to

$$\Delta W - \sigma_f D S = (\Delta\sigma/2\mu)M_0$$

where $\Delta\sigma$ is the stress drop $\sigma_0 - \sigma_1$. Since $\sigma_f D S$ is heat loss during faulting, the left-hand side of this equation gives the total strain energy minus heat loss. Therefore $(\Delta\sigma/2\mu)M_0$ can be considered as the energy available for generation of seismic waves. The stress drop $\Delta\sigma$ is known to be nearly constant for most great and large earthquakes (see for example ref. 13) and $(\Delta\sigma/2\mu)$ is approximately 2×10^{-4} . Therefore $M_0/(2 \times 10^4)$ gives an estimate of seismic energy E . Thus Fig. 2 can be considered as representing the temporal variation of seismic energy release. Note that the energy release in the 1960 Chilean earthquake is 10^{26} erg, which is about 60 times larger than the

value estimated from M_s .

Since the magnitude scale has been used for nearly 40 yr not only among seismologists but also by the news media, it is convenient to express the 'size' of an earthquake in terms of a magnitude scale. To this end, the energy $M_0/(2 \times 10^4)$ can be converted into a magnitude scale by using the energy-magnitude relation, $\log E = 1.5 M + 11.8$. A new magnitude scale, M_w is defined by¹²:

$$M_w = (\log M_0/1.5) - 10.7 \quad (M_0 \text{ in dyn cm})$$

In this scheme, the magnitude is defined in terms of the energy whereas in the old scheme, the energy is calculated from the magnitude. On this scale, $M_w = 9.5$ for the 1960 Chilean earthquake, 9.2 for the 1964 Alaskan earthquake, 9.1 for the 1957 Aleutian Island earthquake, and 9.0 for the 1952 Kamchatka earthquake. In Fig. 2, the M_w scale is given on the right. Comparison of M_w with M_s or M_L shows that M_w agrees very well with M_s and M_L for earthquakes with a smaller fault dimension; thus the M_w scale is a convenient extension of the old magnitude scale to great earthquakes.

Fig. 4 shows the spatial distribution of large earthquakes for the period 1904 to 1976. The values of M_w are given in the bracket for the ten largest earthquakes. It is notable that most of these earthquakes occurred along the Circum-Pacific subduction zones. These ten earthquakes account for more than 90% of the total seismic energy released from 1904 to 1976.

Although this estimate of the energy release of great earthquakes is much larger than that estimated earlier from the magnitude scale, it is still relatively small compared with the average rate of heat flow from the Earth's interior, 7×10^{27} erg yr⁻¹. The average annual rate of the seismic energy release is 4.5×10^{24} erg yr⁻¹, which is about three orders of magnitude smaller than the heat flow. Even for the largest event, the 1960 Chilean earthquake, the seismic energy is 10^{26} erg, which is about 1.4% of annual heat flow. However, the total strain

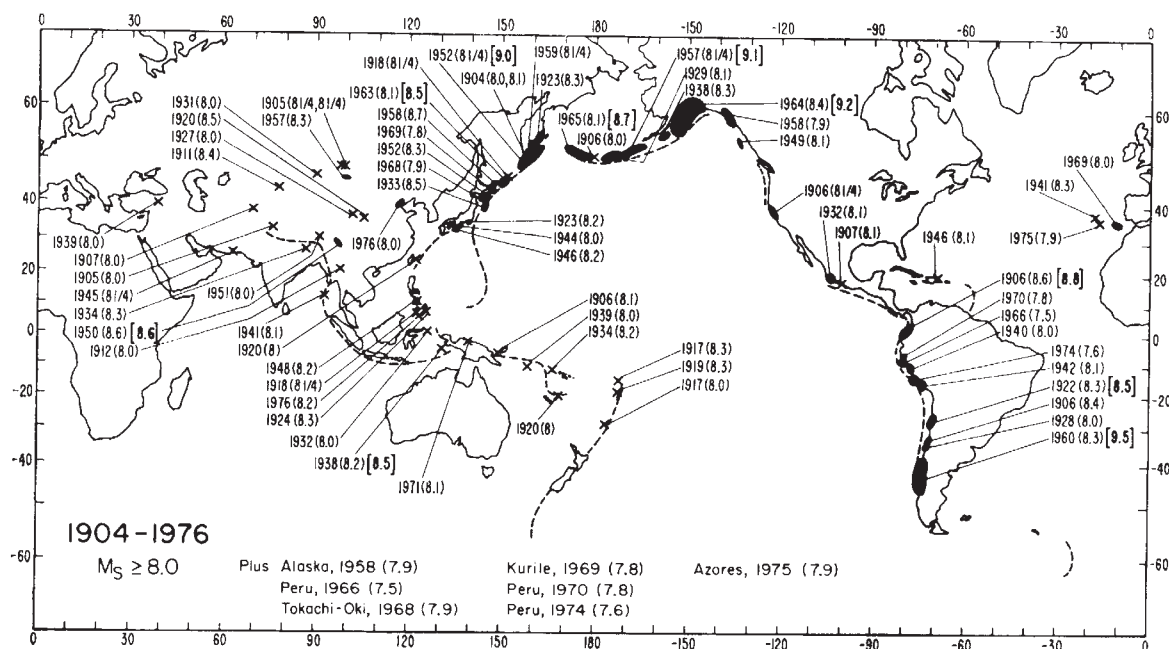


Fig. 4 Great and large earthquakes for the period from 1904 to 1976. The surface-wave magnitude M_s is given in the parentheses and M_w is given in the brackets for the ten largest earthquakes. Major rupture zones are indicated by dark zones.

energy change associated with an earthquake can be much larger (100 times more) than the seismic energy, if the stress drop in earthquakes is only a small fraction of the ambient tectonic stress. The discrepancy between the stress drop in earthquakes (10–100 bar) and the strength of rocks measured in the laboratory (several kilobar) suggests this possibility, but the problem remains unresolved up to now. If this is the case, the total energy involved in the earthquake process can be very significant in the energy budget of the Earth.

Since the total crustal deformation associated with great earthquakes is very large, the study of great earthquakes has a direct bearing on various global problems such as the Chandler wobble, plate motion and the rotation of the Earth^{14–16}. For the moment, the data and analysis are not complete enough to study this problem in detail, but with recent improvements in

instrumentation¹⁷ many important problems in this field may be resolved in the near future.

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articles

Structures and molecular motions in alkaline earth hexammines

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The alkaline earth hexammines have novel structures and molecular motions. The ammonia molecules in these compounds adopt an entirely different geometry from that of normal ammonia. There are two motional transitions as the temperature is increased. The first transition is due to ammonia rotation, and the second results from ammonia diffusion.

STRUCTURE and molecular motion in solids receive a great deal of experimental and theoretical attention and we have initiated a comprehensive research programme to investigate the compounds formed by reaction of elemental metals with ammonia. Only six metal-ammonia compounds are known: $\text{Li}(\text{NH}_3)_4$ and $\text{M}(\text{NH}_3)_6$, where $\text{M} = \text{Ca}, \text{Sr}, \text{Ba}, \text{Eu}, \text{or Yb}$ (ref. 1). All these compounds are metals, and their metallic nature originates from the loss of one, in $\text{Li}(\text{NH}_3)_4$, and two, in $\text{M}(\text{NH}_3)_6$, valence electrons from the elemental metal to a

Table 1 Lattice constants and ^1H NMR parameters

Compound	$a_0(\text{\AA})$	$T_w(\text{K})^*$	$T_s(\text{K})^\dagger$	$E_a(\text{k cal mol}^{-1})$	$\nu_0(\times 10^{-11}\text{Hz})$
$\text{Ca}(\text{NH}_3)_6$	9.01	62	102	3.2	3.3
$\text{Sr}(\text{NH}_3)_6$	9.50	< 40	97	2.7	0.5
$\text{Ba}(\text{NH}_3)_6$	9.73	< 20	73	2.1	1.0

*Onset temperature for the weak-narrowing transition.

†Onset temperature for the strong-narrowing transition.

conduction band. The motivation for our research is the belief—supported by electrical transport^{2–4}, magnetic^{5,6}, and magnetic resonance⁷ experiments—that these compounds are low-electron-density metals, and as such are rare model systems for testing theories of the electronic behaviour of solids at low electron densities. In addition to the unique electronic properties of metal-ammonia compounds, however, we have discovered that they exhibit both novel structures⁸ and molecular motions⁹. Here we report and interpret the results of our structural and motional studies on the alkaline earth hexammines $\text{M}(\text{NH}_3)_6$, where $\text{M} = \text{Ca}, \text{Sr}, \text{or Ba}$.

The structures of the deuterated compounds have been examined by powder neutron diffraction at 75 K (ref. 8). The sample-preparation technique and neutron spectrometer have been described elsewhere⁸. The hexammines all crystallise in a body-centred-cubic structure. The lattice constants are given in Table 1. To date, only $\text{Ca}(\text{ND}_3)_6$ has been examined in detail, and the atomic parameters were refined by a least-squares fitting of the diffraction profile. $\text{Ca}(\text{ND}_3)_6$ crystallises in the space group $\text{Im}\bar{3}\text{m}$ and has molecular complexes, $\text{Ca}(\text{ND}_3)_6$, located at each lattice site. The six nitrogens in a complex ion are arranged in an exact octahedron around the calcium, with a rather long Ca–N distance of 2.69 Å. The ammonia molecules in $\text{Ca}(\text{ND}_3)_6$ have an entirely different geometry from that of normal ammonia, where the N–H bond angle and distance are 110° and 1.00 Å, respectively. In $\text{Ca}(\text{ND}_3)_6$, the ammonia molecules are nearly planar and have two inequivalent sets of deuterons, with one N–D distance being short (0.94 Å), while the other two are extremely long (1.39 Å). Further evidence for this ammonia structure is provided by the low-temperature proton magnetic resonance (^1H NMR) experiments discussed below. Although each ammonia molecule is coordinated to the calcium by the nitrogen, the pseudotrigonal axis of each ammonia molecule is not coincident with the Ca–N bond, but makes an angle of 13° with it. Furthermore, each ammonia molecule has a 4-fold rotational disorder at 75 K, so that there are a total of 4⁶ possible ammonia orientations in each complex ion. One of the possible structures of the complex is shown in Fig. 1. In addition, there is no hydrogen bonding in the structure and virtually all nonbonded contacts are greater than the van der Waals distances, so that there are essentially no restrictions on the possible ammonia and complex rotations. The temperature factor is 7.4 Å², which indicates considerable thermal motion at 75 K. Although the structures of the other two hexammines have not yet been refined, we anticipate that the hexammines are isomorphous as their unit cells, lattice parameters, ^1H NMR, and chemical behaviour are nearly the same.

Molecular motions

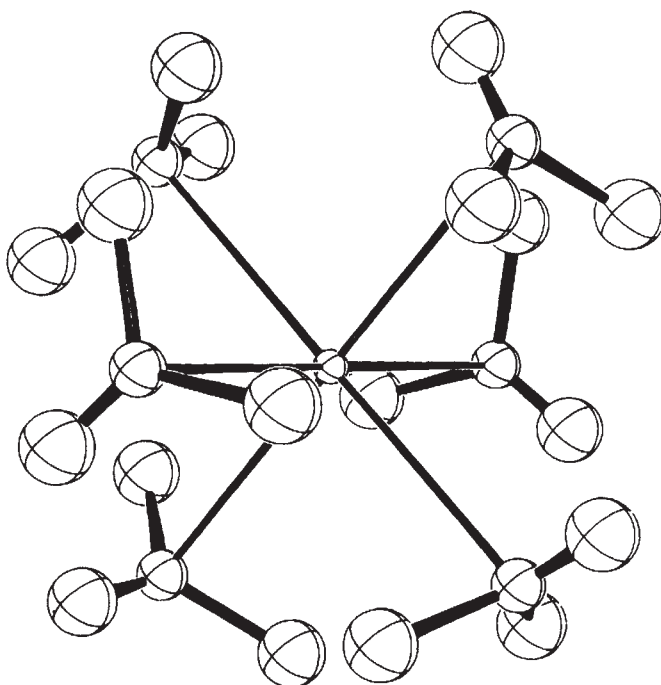
Molecular motion in the alkaline earth hexammines has been investigated by ^1H NMR. The sample-preparation technique and wide-line NMR spectrometer have been described elsewhere¹⁰. The spectra consist of a single asymmetrical line, as expected for metals having dimensions much larger than the skin depth, and the asymmetry parameters all lie in the range 1.4–1.9. The temperature dependence of the first-derivative peak-to-peak linewidth, ΔH , is shown in Fig. 2. In $\text{Sr}(\text{NH}_3)_6$, the signal was too weak to detect below 40 K. It is clear from Fig. 2 that all the hexammines exhibit the same qualitative behaviour. In particular, the low-temperature linewidth is about

2 G and, as the temperature is increased, there are two line-narrowing transitions. At the first transition there is a weak narrowing and at the second a very strong narrowing of the ^1H NMR line. The onset temperatures for these transitions are shown in Table 1. The strong-narrowing transitions are completed by 140 K. Above 140 K, the linewidth is independent of temperature and equal to 35 mG. But, because 35 mG is the magnetic-field inhomogeneity on our wide-line spectrometer, high-resolution ^1H NMR experiments were performed to determine the true linewidth. A Varian XL-100-12 NMR spectrometer, equipped with variable-temperature accessory, was used to record high-resolution ^1H NMR spectra in the range 190–300 K. A deuterium lock was employed by placing acetone- d_6 in the outer volume of a coaxial NMR tube (o.d. = 5 mm, i.d. = 3 mm). The measured full-width at half-maximum absorption is independent of temperature in the hexammines and equal to 15 mG. As the hexammines melt near 273 K, the linewidth does not change upon melting. Furthermore, the high-resolution linewidth in liquid ammonia just above the melting point is 11 mG, which is only 4 mG narrower than the hexamine linewidth above 140 K. Below we interpret the ^1H NMR linewidth results in the hexammines in terms of structure and molecular motion.

Linewidth results in hexammines

Using the interproton distances derived from neutron diffraction, the calculated rigid-lattice peak-to-peak linewidth is about 6 G (ref. 11), which is in poor agreement with the low-temperature linewidth of about 2 G. But if, by analogy to solid ammonia¹², the ammonia molecules in the hexammines tunnel at low temperature, then the estimated linewidth is about 3 G (the low-temperature linewidth was estimated by assuming that the intra and inter-ammonia contributions to the second moment are reduced by factors of $\frac{1}{4}$ and $\frac{1}{2}$, respectively, as found for these contributions in solid ammonia at low temperatures¹²), which is in reasonable agreement with experiment. This result supports the neutron-diffraction structure of

Fig. 1 One of the 4⁶ possible structures of the molecular complex $\text{Ca}(\text{ND}_3)_6$ in metallic $\text{Ca}(\text{ND}_3)_6$. The central calcium is octahedrally coordinated to the nitrogens of the six surrounding ammonia molecules.



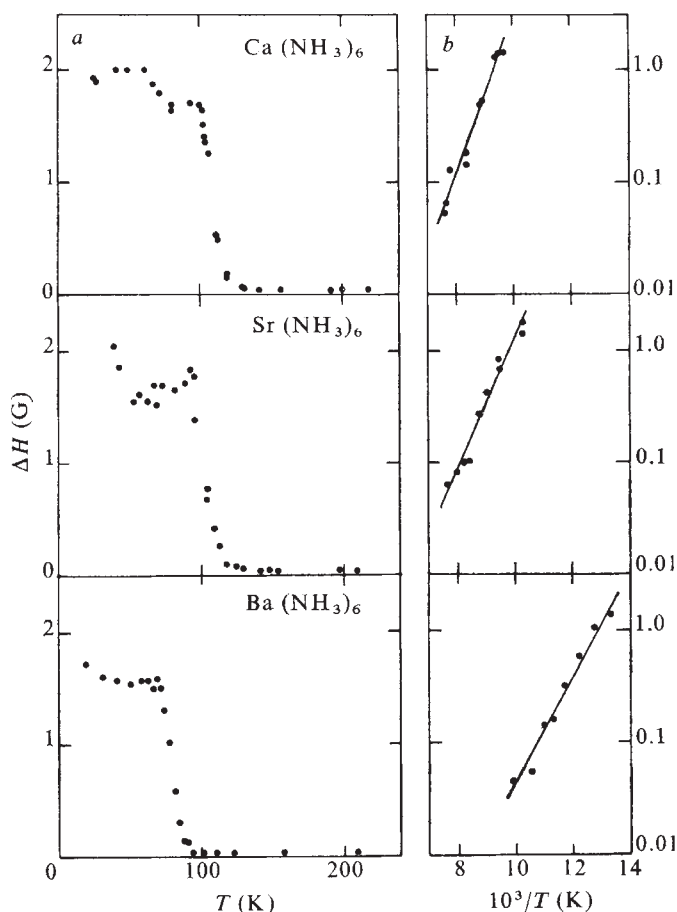


Fig. 2 Temperature dependence of the linewidth (a) and semi-logarithmic plot of linewidth versus reciprocal temperature (b) for the alkaline earth hexammines.

the hexammines as the calculated low-temperature linewidth, using the structural parameters for an ammonia molecule in solid ammonia¹³ and assuming tunnelling, is about 8 G which is a factor of 4 larger than the experimental value.

We attribute the weak-narrowing transition to a transition from quantum-mechanical tunnelling to classical rotation of ammonia molecules. A similar weak-narrowing transition has been observed in solid ammonia between 60 and 80 K, and it was shown to result from the aforementioned rotational transition¹². Further indication of this rotational transition below 77 K in metal-ammonia compounds comes from changes in the electrical resistivity of $\text{Li}(\text{NH}_3)_6$ (ref. 14) and the Mössbauer resonant absorption in $\text{Eu}(\text{NH}_3)_6$ (ref. 15) near 70 K. The decrease in T_w with increasing molecular weight is correlated with a corresponding increase in cell volume (see Table 1); this should lower the barrier to rotation.

The strong-narrowing transition is indicative of other types of proton motion in addition to that associated with ammonia rotation. From the high-resolution ^1H NMR results, it follows that above the strong-narrowing transition the protons diffuse rapidly on the ^1H NMR time scale. Assuming that proton diffusion is a thermally activated process, we used the Gutowsky-Pake equation to analyse the linewidth data for the strong-narrowing transition:

$$\ln \Delta H = E_a/RT + \ln \alpha \gamma (\Delta H_{\text{RL}})^2 / \pi^2 \nu_0$$

where E_a is the activation energy, α is a constant of the order of unity, ΔH_{RL} is the rigid-lattice linewidth, and ν_0 is the vibrational frequency of the jumping atom or molecule¹⁶. (This equation is valid provided that $(\Delta H_{\text{RL}})^2 \gg (\Delta H)^2 \gg (\Delta H_{\text{HT}})^2$, where ΔH_{HT} is the residual high-temperature line-

width. The above inequalities hold over most of the strong-narrowing transition.) The jump frequency is given by $\nu_i = \nu_0 \exp(-E_a/RT)$, and the reciprocal of the jump frequency is the correlation time, τ_c (ref. 17). As shown in Fig. 1, the semi-logarithmic plot of ΔH against T^{-1} is linear. Taking $\alpha = 1$ and $\Delta H_{\text{RL}} = 6$ G, the calculated values of E_a and ν_0 are summarised in Table 1. Also $\tau_c = 3.5 \times 10^{-6}$ s when $\Delta H = 0.2$ G, and τ_c decreases exponentially with increasing temperature. The low values of E_a and τ_c are consistent with rapid proton diffusion above 140 K. Also, it is interesting that ν_0 is in order-of-magnitude agreement with that expected for the metal-ammonia symmetric-breathing vibration¹⁸.

Mechanisms of proton diffusion

We have considered the following mechanisms of proton diffusion as the most likely possibilities: (1) isotropic reorientation of $\text{M}(\text{NH}_3)_6$ complexes, (2) diffusion of $\text{M}(\text{NH}_3)_6$ complexes, (3) proton exchange alone, and (4) diffusion of NH_3 . We have calculated that mechanism (1) results in a linewidth of about 1 G (noting that the effect of isotropic reorientation is to concentrate the 18 protons of a complex at the metal site, the linewidth was calculated by summing over the four nearest shells of complex ions and then integrating to estimate the remaining contribution. Nearest-neighbour interactions account for 85% of linewidth), so that it could account for only a small fraction of the observed narrowing. Although mechanism (2) would further narrow the line, we consider this process unlikely in view of the large diameter and mass of the $\text{M}(\text{NH}_3)_6$ complex. As T_s and E_a decrease with increasing molecular weight (see Table 1), it supports the contention that mechanism (2) is unimportant. As virtually all nonbonded contacts are greater than the van der Waals distances, mechanism (3) cannot occur by a rotational mechanism. The only other possibility is for (3) to break the N-H bond. However, molecular-orbital calculations have indicated that the N-H bonds in the hexammines are comparable in strength to those in ammonia (INDO calculations indicate that an ammonia molecule in the hexammines is less stable than normal ammonia by about 5 kcal mol⁻¹) so, as in ammonia, proton exchange does not occur until ammonia diffusion sets in.

We conclude, therefore, that the strong-narrowing transition in the hexammines is due to mechanism (4). (Intermolecular proton exchange accompanies proton diffusion in ammonia. It has been estimated, however, that the correlation time for such proton exchange is greater than 10^{-4} s (ref. 19), which is several orders of magnitude larger than the experimental correlation times. Hence the rate of proton exchange is very slow compared to that of ammonia diffusion, so that the integrity of the ammonia molecule is maintained above the strong-narrowing transition.) The ^1H NMR results are all consistent with this. The structural basis for ammonia diffusion is provided by the rather long M-N distance and nonstoichiometry²⁰. The long M-N bond is probably weak and hence can be broken easily, which accounts for the low transition temperatures and activation energies, and nonstoichiometry, in the form of ammonia vacancies, provides available sites to which the ammonia molecules can jump. The strong-narrowing transition may reflect a melting of the ammonia sublattice far below the melting point of the metal, and we believe this would be the first example of molecular-sublattice melting in solid state science. We are investigating this possibility using pulsed-proton NMR and inelastic neutron scattering to elucidate further the details of the motional transitions in the alkaline earth hexammines.

Finally, we shall comment upon the activation energies in Table 1. The experimental activation energies include the M-H bond energy as well as the energy required to transport ammonia, so that they are an upper limit to the N-H bond energy. The decrease in activation energy with increasing molecular weight follows from the corresponding increase in cell volume, which

increases the void volume available for ammonia diffusion and hence lowers the activation energy for transport, and also the expected corresponding decrease in bond energies, which lowers the activation energy for bond breaking.

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Nucleotide sequence of the origin of replication in bacteriophage Φ X174 RF DNA

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The gene A protein of bacteriophage Φ X174 has been used in vitro to convert Φ X RFI DNA into the relaxed RFII form by nicking the viral strand. The nucleotide sequence at the 3' end of the nick has been determined as --TGC TCCCCCAACTTG_{OH}. This sequence gives the exact position of the origin of Φ X RF DNA replication.

THE replication of the bacteriophage Φ X174 RF DNA and the way in which the viral gene A is involved in this process has been studied in great detail both *in vivo* and *in vitro*. Results of many workers show that the Φ X174 RF DNA replication starts by nicking the viral strand of the parental RFI DNA which is then elongated from the 3'-OH end under simultaneous displacement of the 5' end, using the circular complementary strand as a template. Therefore the 3'-OH end of the nick is the origin of Φ X RF DNA replication. This rolling circle mode of DNA replication was originally proposed by Gilbert and Dressler¹. The synthesis of a new complementary strand on the displaced viral strand occurs in small DNA fragments and therefore has no unique initiation site^{2,3}.

The location of the origin of replication has been investigated extensively *in vivo*. Repair studies with heteroduplex DNA showed the origin to be within gene A (ref. 4). Using *rep*⁻ cells, the position of the nick in the viral strand of Φ X RFI could be located with high precision in the *Hae*III restriction fragment Z6_B, approximately 185 base pairs away from the Z6_A/Z6_B junction⁵. The termination site for Φ X DNA replication has also been placed in this region of the Φ X genome⁶⁻⁸.

The gene A product of the phage is an absolute requirement for the RF DNA replication *in vivo*⁹. In their studies of Φ X DNA replication in *rep*⁻ cells, Francke and Ray^{10,11} showed that part of the parental RFI molecules was nicked in the viral strand only if a functional gene A product is present. Furthermore the gene A product was shown to be *cis*-acting, that is, in simultaneous infections with phage with an intact gene A and phage with a mutated gene A, only the RF derived from the phage with an intact gene A acquired a discontinuity in the viral strand. This is consistent with the asymmetrical

complementation behaviour of gene A mutants⁹. The gene A protein has been isolated since then¹²⁻¹⁴. It is a 55,000 molecular weight (MW) protein and it has a specific endonuclease activity. The A protein nicks Φ X RFI DNA once and in the viral strand only¹² and more precisely within gene A in the *Hind*III fragment R3 (ref 14). These properties of the gene A protein clearly connect the 3' end of the nick made by the A protein with the 3' end that is the origin of RF DNA replication. The A protein fulfils all the requirements of the initiator protein in the replicon model proposed by Jacob, Brenner and Cuzin¹⁵. It is coded for by the viral replicon, it is required before DNA replication has started and it produces a free 3'-OH end that serves as a primer in the subsequent elongation of the viral strand.

We describe here experiments with the purified A protein and the determination of the nucleotide sequence at the 3' end of the nicked viral strand of RFI DNA that has been incubated with A protein. The sequence found identifies the site of the origin of replication unequivocally in the known nucleotide sequence of Φ X174 (ref. 16).

Gene A protein

THE A protein was isolated from *Escherichia coli* HF4704 (*uvrA*, *thy*, *sup*⁻) cells that had been infected with the lysis-defective phage mutant *am3*. The enzyme purification followed the procedure of Eisenberg *et al.*¹³ with the exception that no chloramphenicol was used during infection. This modification greatly improved the yield of the A protein. The enzyme preparation obtained after DNA-cellulose column chromatography (Fig. 1) contains the two proteins that are coded for by the gene A (ref. 17) (the 55,000 MW A protein and the 37,000 MWA* protein which has no nicking activity¹⁴). This protein mixture was used in the experiments on the endonucleolytic activity of the A protein.

The endonucleolytic activity of the A protein preparations was measured by the conversion of supertwisted covalently closed replicative form DNA (RFI) to the relaxed form containing at least one single-strand nick (RFII). This conversion was visualised on 0.6% agarose gels containing ethidium bromide¹⁶ by the difference in mobilities of the RFI and RFII

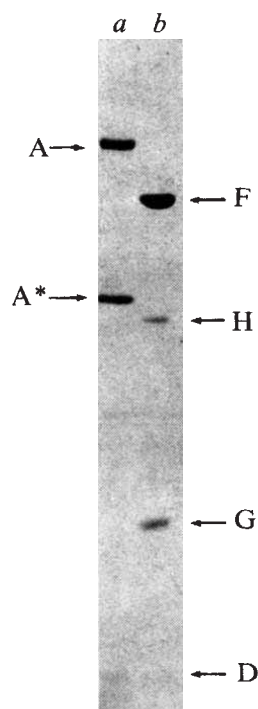


Fig. 1 Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis of the A protein preparation. The A protein was purified from 15 g *E. coli* HF4704 infected with Φ X174 *am3* for 30 min, following the procedure of Eisenberg *et al.*¹³. Cells were lysed with lysozyme at 37 °C. Solid NaCl was added to a concentration of 1 M and cell debris was removed from the lysate by low speed centrifugation. Ammonium sulphate was added at 0.226 g per ml supernatant over a period of 20 min. After 15 min the precipitate was collected by centrifugation and resuspended in 50 mM imidazole-HCl, pH 6.7, 10 mM β -mercaptoethanol, 1 mM EDTA, 20% glycerol and 1 M NaCl and fractionated on a Biogel A 0.5 m column (150 ml) equilibrated in the same buffer. Proteins eluted from the Biogel column were applied to a Biorex 70 column (15 ml) in buffer containing 0.3 M NaCl. The column was washed with at least four column volumes and finally washed with buffer containing 1 M NaCl. The 1 M NaCl eluate was diluted to 0.4 M NaCl and passed through a single-stranded DNA-cellulose column (2 ml). The A protein was eluted from the column with 1 M NaCl. Each step in the purification was followed by electrophoretal analysis of samples on 12.5% polyacrylamide slab gels prepared according to Laemmli³⁶. *a*, The final A protein preparation after the DNA-cellulose column. *b*, The marker proteins are the phage proteins F, H, G and D with molecular weights of 48,000, 36,000, 22,000 and 14,500 respectively³⁷.

DNA molecules. The amount of RFI DNA converted to RFII DNA was dependent on the amount of A protein but never exceeded 50%. Whether this is the consequence of the presence of the A* protein or not, is unknown. No linear double-stranded DNA (RFIII) was formed, even after incubation with a 10-fold excess of A protein (compared with the minimal amount of A protein needed for 50% conversion). When incubation times were increased to 2 h, in some experiments a minor DNA band was formed that migrated with the mobility of RFI DNA containing no supertwists (relaxed RFI¹⁹). Relaxed RFI DNA formed during incubation of RFI DNA with A protein has also been found by Ikeda *et al.*¹⁴.

Incubation of the reaction mixture with proteinase K before phenol extraction is required for quantitative recovery of newly formed RFII DNA. The strong interaction of the A protein with the DNA causes the extraction of the DNA together with the protein out of the water phase.

The strand specificity of the enzyme activity was tested on RFI DNA labelled with ³H-thymidine in the complementary (—) strand only. Alkaline sucrose gradient analyses of the RFI DNA before and after incubation of this RFI DNA with the A

protein were carried out. The untreated RFI DNA (Fig. 2*a*) sediments much faster than single-stranded DNA. Figure 2*b* shows that after incubation of this RFI DNA with A protein almost all radioactivity in the RFII DNA formed is found after denaturation as circular single-stranded DNA. No increase in label is found at the position of linear single-stranded DNA. This indicates that the A protein nicks only the viral strand of RFI DNA (see also refs 12 and 14).

Site of cleavage

Attempts to label the 3' end of the nick made by the A protein in the viral strand of RFI DNA with terminal transferase²⁰ in the presence of Co²⁺ were unsuccessful. Also treatment of the RFII DNA with bacterial alkaline phosphatase followed by incubation of the DNA with T4 polynucleotide kinase²¹ did not result in incorporation of label at the 5' end. To circumvent the problems with intact RFII DNA, we determined first after digestion with the restriction enzyme *Hae*III in which restriction fragment the nick was located. This nicked fragment was purified and separated into its single-stranded components. The single-stranded viral (+) strand fragments were labelled at their 3' end and the nucleotide sequence at the site of the nick was determined. We were unable to label the 5' end of the nick.

³²P-labelled RFI DNA was digested with the A protein in conditions as described in Fig. 3. The mixture of RFI and RFII DNA was then digested to completion with restriction endonuclease *Hae*III. The digestion mixture was deproteinised by incubation with proteinase K followed by phenol extraction and the DNA fragments were separated on a 5% polyacrylamide slab gel. The separation of the fragments was visualised by autoradiography. The separate DNA bands were excised and the fragments eluted from the gel. All fragments were subsequently electrophoresed on slab gels in conditions in which all secondary structure of the DNA is disrupted²². The two strands of each restriction fragment are not separated in this gel system. Only the double-stranded fragment that contains the nick yields three single-stranded fragments of different size and mobility. This is shown in Fig. 3, the autoradiograph of the electrophoreses in denaturing conditions of the fragments Z6_B, Z7, Z8, Z9 and Z10. Z7, Z8, Z9 and Z10 give a single band but Z6_B is separated into three different fragments, approxi-

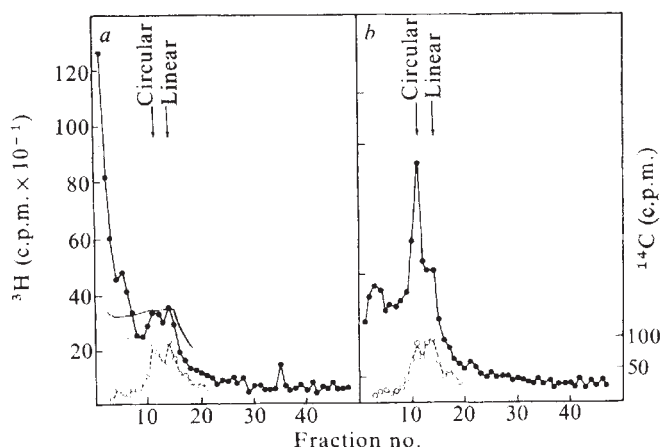


Fig. 2 Alkaline sucrose gradient analysis of RFI DNA treated with A protein. 1.25 μ g RFI ³H-labelled in the complementary strand (●) was incubated with 6 μ g A protein in 50 mM imidazole-HCl at pH 7.0, 60 mM NaCl, 10 mM MgCl₂ and 10 mM β -mercaptoethanol in a volume of 125 μ l at 37 °C for 30 min. Proteinase K (10 μ g), preincubated for 30 min at 37 °C, was added and the incubation was prolonged 20 min at 37 °C followed by extraction with phenol. The DNA samples were layered on 10–30% alkaline sucrose gradients (Baas *et al.*⁵) and centrifuged in the SW41 rotor for 24 h at 38,000 r.p.m. at 15 °C. ¹⁴C-labelled (○) single-stranded linear and circular DNA was added as markers. *a*, RFI DNA without A protein. *b*, RFI DNA after incubation with A protein.

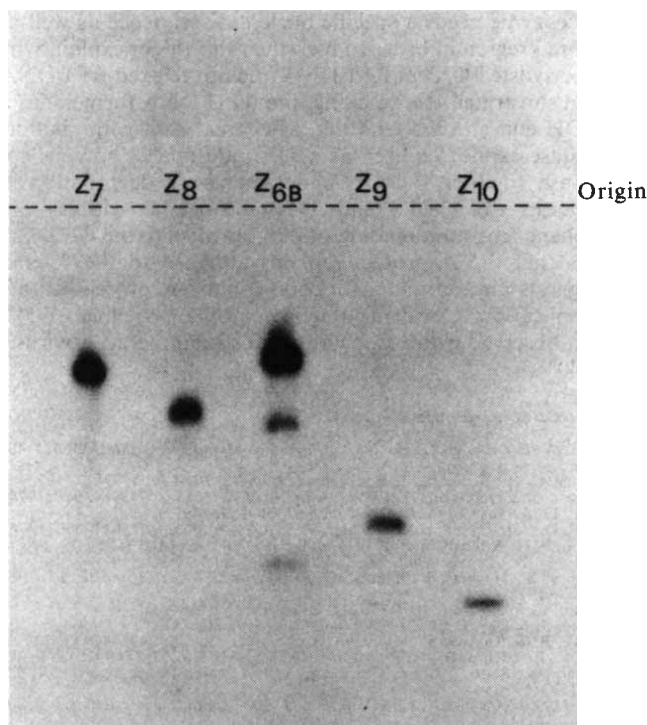


Fig. 3 Autoradiograph of gel electrophoresis in denaturing conditions of the *Hae*III fragments Z_{6B}–Z₁₀. 400 µg RFI DNA was mixed with 50 µg ³²P-labelled RFI (4.6 × 10⁶ c.p.m. Cerenkov) and incubated with 640 µg A protein in the conditions described in Fig. 2 legend. The final volume was 3.2 ml. The DNA was digested with *Hae*III followed by proteinase K treatment and phenol extraction. Fragments were separated on a 5% polyacrylamide gel that had been cross-linked with 0.3% ethylenediacrylate in 50 mM Tris-borate, 1 mM EDTA pH 8.35, which was also the running buffer. Extraction of the DNA fragments was done by dissolving the gel in 0.5 M ammonium carbonate, 0.1 mM Mg acetate, 0.1% SDS and 100 mM EDTA at pH 9.2 for 16 h at 37 °C. Samples containing the fragments were diluted 20-fold with water and percolated through a hydroxyapatite column. Fragments were eluted with 0.4 M K phosphate, pH 6.8. The recovery was close to 100% for all fragments. The DNA fragments were alcohol-precipitated and dissolved in 20 µl formamide containing 20% sucrose, 0.1% xylene cyanol and 0.1% bromophenol blue and were incubated at 100 °C for 3 min. The longer fragments (Z₁–Z_{6A}) were layered on a 4% polyacrylamide gel made up in 98% formamide (not shown) and the shorter fragments (Z_{6B}–Z₁₀) on a 10% polyacrylamide gel made up in 98% formamide. The gels were made up as described by Maniatis *et al.*²³. The length of the fragments in nucleotides is: Z_{6B} 278; Z₇ 234; Z₈ 194; Z₉ 118 and Z₁₀ 78 (ref. 16).

mately 280, 180 and 100 nucleotides in length. The 280 nucleotides band consists of the complementary strand and unnicked viral strand DNA of fragment Z_{6B}. Fragments Z₁ to Z_{6A} give only a single band (data not shown).

From this result we conclude that the nick introduced by the A protein in the viral strand of RFI DNA, is located in the *Hae*III fragment Z_{6B} and cleaves the viral strand of Z_{6B} in pieces of 180 (Z_{6B2}) and 100 (Z_{6B3}) nucleotides each.

The single-stranded fragments Z_{6B2} and Z_{6B3} were eluted from the gel and used for endgroup labelling. Incubation of fragment Z_{6B3} with terminal transferase and α-³²P-UTP in the presence of Co²⁺ in order to label the 3' end²⁰, resulted in incorporation of ³²P label (for details see Fig. 4 legend). The ³²P-labelled Z_{6B3} fragment was partially digested with micrococcal nuclease and spleen phosphodiesterase. The products were separated on the standard two-dimensional system²³ and after autoradiography the pattern presented in Fig. 4 was found. From the mobility shift of the labelled oligonucleotides^{24,25} the sequence of the 3' terminus was determined. The 3' terminal sequence of the fragment Z_{6B3} therefore is -T G C T C C C C C A A C T T G.

The last two nucleotides from the 3' terminus are not part of the *Hae*III recognition site (G G C C) and therefore this 3'

end must have been generated by the A protein. The fact that the 3' end has been extended by terminal transferase, which requires a free 3'-OH, indicates that the terminal G has a free 3'-OH group.

From these experiments we infer that the 5' end at the site of the nick is the same as the 5' end of the fragment Z_{6B2}. Experiments in which we tried to label this 5' end by treating Z_{6B2} with alkaline phosphatase followed by incubation with T4 polynucleotide kinase and γ-³²P-ATP were unsuccessful. This fact together with the observation that the A protein binds tightly to RFI DNA points towards an interaction of the A protein with the 5' end such that this end is no substrate for phosphatase and/or kinase. The presence of a 3'-OH group and a blocked 5' end at the nick has also been suggested by Ikeda *et al.*¹⁴.

Conclusions

The nucleotide sequence found at the 3' end of the endonucleolytic scission made by the gene A protein in the viral strand of RFI DNA has been determined. The nick is located in the *Hae*III fragment Z_{6B} within gene A²⁸. A comparison with the complete sequence of ΦX174 DNA¹⁶ shows that the sequence determined is unique in ΦX174 and corresponds to the nucleo-

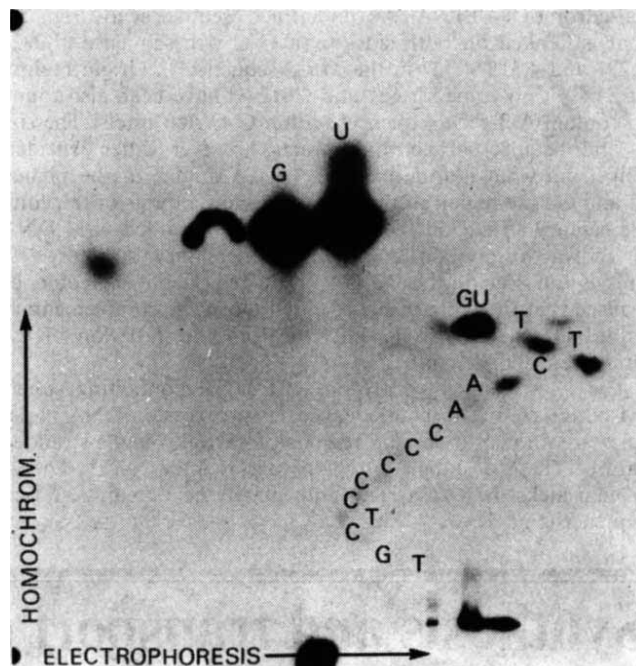


Fig. 4 Autoradiograph of the two-dimensional separation of the oligonucleotides obtained after partial digestion of 3' labelled fragment Z_{6B3}. The DNA fragment Z_{6B3} was eluted from the gel shown in Fig. 3 and labelled with terminal transferase and [α-³²P]UTP in the presence of Co²⁺ by the method of Roychoudhury *et al.*²⁰. Then it was treated with alkali for 16 h at 37 °C, to remove all but the first transferred U residue. When this alkali treated material was incubated with alkaline phosphatase 50% of the ³²P label was removed from the DNA fragment indicating that at least two U residues had been added to each DNA molecule during the terminal transferase reaction. Analysis of a single-stranded DNA fragment with a known 3' end with terminal transferase showed that the terminal transferase was free of exonucleolytic activity. The alkali treated material was alcohol-precipitated, dissolved in 10 mM Tris-HCl pH 9.0, 2.5 mM CaCl₂ and incubated with micrococcal nuclease at 37 °C. After 15 min the pH was lowered to pH 5.0 with HCl and spleen phosphodiesterase was added. The required amounts of micrococcal nuclease and spleen phosphodiesterase and incubation times were determined in pilot experiments. The digestion products were separated in the standard two-dimensional system²³, using electrophoresis on cellulose acetate at pH 3.5 in the first direction and homochromatography on DEAE thin layer using a 3% homomix in the second direction. The oligonucleotides were visualised by autoradiography. Starting from the dinucleotide GU the nucleotide causing the shift of the next longer oligonucleotide is indicated.

peptides in the nuclear genome^{6,7}. It is thus clear that the formation of chloroplasts requires the integrated activities of both chloroplast and nuclear genomes. Current interest lies in defining the precise contribution of each genetic system and the mechanism of their interaction. Why do chloroplasts contain so many ribosomes, and how do those chloroplast polypeptides made by cytoplasmic ribosomes enter the developing organelle?

Recent technical advances have allowed the isolation of intact chloroplasts which use light energy to drive the synthesis of discrete protein and RNA molecules^{1,8,9}. Analysis of the products of protein synthesis in isolated pea chloroplasts by electrophoresis on one- and two-dimensional polyacrylamide gels has revealed two major products and about 90 minor products¹⁰. One major product is tightly bound to the chloroplast lamellae, and is associated with the ATP-synthase complex¹⁰. The other major product is soluble, and has been identified as the large subunit of ribulose biphosphate carboxylase/oxygenase¹¹, the key enzyme in photosynthesis and photorespiration. This carboxylase (RBPCase) comprises up to 50% of the total soluble protein in leaves, and thus may be the most abundant protein in nature¹². The unusual abundance of this protein could account for the high proportion of ribosomes found inside chloroplasts. The RBPCase molecule is an oligomer of 16 subunits; 8 are termed large (molecular weight (MW) ~55,000) and carry the catalytic sites, while the other 8 are termed small subunits (MW ~14,000). The large subunit is encoded in chloroplast DNA¹³, and is synthesised from a messenger RNA lacking poly A (ref. 14). By contrast, the small subunit is encoded in nuclear DNA¹³; this subunit is not labelled when isolated chloroplasts incorporate labelled amino acids into protein but it has been identified as an *in vitro* translation product of cytoplasmic ribosomes¹⁵. We report here that the small subunit is synthesised as a precursor of higher molecular weight (20,000) when poly A-RNA from pea cytoplasmic ribosomes is translated by a heterologous protein-synthesising system. This precursor enters intact isolated chloroplasts with cleavage to the final size. In direct contrast to other systems where proteins cross membranes^{16,17}, this uptake and cleavage does not require concomitant protein synthesis. We propose that polypeptides enter the chloroplast after synthesis by a mechanism involving combination with specific sites in the chloroplast envelope.

Synthesis of precursor to the small subunit

When seeds of the pea plant (*Pisum sativum*) are germinated in darkness, the etiolated seedlings that result contain small amounts of RBPCase. If such seedlings are exposed to light, this enzyme accumulates rapidly as part of the development of chloroplasts that ensues¹⁸. Polysomes were prepared from etiolated pea apices after exposure to light for various periods by a method which minimises degradation by nucleases¹⁹. Total RNA was extracted from these polysomes and fractionated on a column of oligo dT-cellulose²⁰. The bound fraction containing poly A-RNA was then translated in the presence of ³⁵S-methionine by a protein-synthesising system prepared from wheat-germ²¹. Analysis of the products by one-dimensional electrophoresis on polyacrylamide gels containing sodium dodecyl sulphate (SDS) revealed many labelled bands. One band shows a marked and continuing rise in amount relative to the other bands when the poly A-RNA is isolated from apices exposed to light for progressively longer periods (Fig. 1). This band is specifically precipitated by rabbit antiserum prepared against purified pea RBPCase, but not by preimmune serum (Fig. 2). This immunoprecipitation is competed out by added RBPCase. Since the messenger RNA for the large subunit of RBPCase does not bind to oligo dT-cellulose in the conditions used¹⁴, we conclude that this band contains the small subunit polypeptide. This band has an apparent molecular weight of 20,000—about 6,000 greater than that of the small subunit prepared from purified pea RBPCase. These observations suggest that the small subunit is translated as a higher molecular weight

precursor from a messenger RNA containing poly A; we term this precursor P20. A similar precursor has been reported for the small subunit of RBPCase from *Chlamydomonas*²².

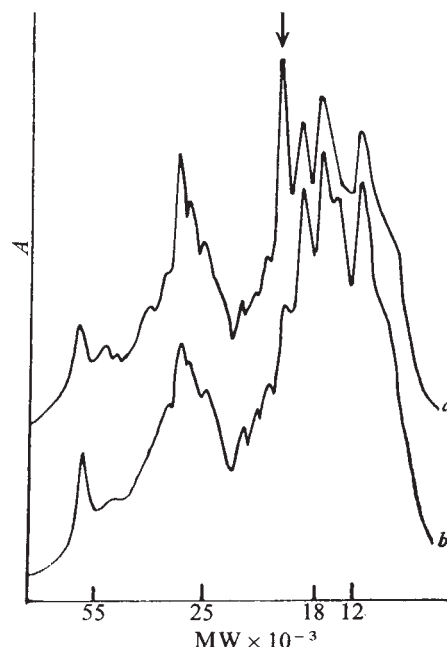


Fig. 1 Products of translation of pea leaf poly A-mRNA. Pea seedlings were grown in the dark for 9 d and then exposed to continuous white light (10,000 lx) for various periods of time. At intervals leaves were frozen in liquid nitrogen and ground to a fine powder. The powder was homogenised in 200 mM sucrose, 200 mM Tris-HCl, 60 mM KCl, 30 mM MgCl₂, pH 8.5 containing 1% (v/v) Nonidet P40 and 0.5% sodium deoxycholate (5 ml for every gram of frozen weight of powder). A polysome pellet was prepared from the homogenate as described previously¹⁹. RNA was extracted from the pellet as described by Brawerman²⁰. Oligo dT-cellulose chromatography was used to obtain poly A-RNA²⁰; LiCl rather than KCl was used because potassium dodecylsulphate comes out of solution readily. The poly A-RNA was used to programme a wheat-germ protein-synthesising system²¹. Each incubation contained in a final volume of 20 µl; 1 mM Tris-ATP, 0.1 mM GTP, 10 mM creatine phosphate, 0.05 mM of each amino acid except methionine, 10 mM dithiothreitol, 110 mM KCl, 2.5 mM Mg acetate, 250 µM spermidine, 50 µM spermine, 8.8 µCi ³⁵S-methionine (800 Ci mmol⁻¹) and 4 µg RNA. The mixture was incubated for 1 h at 27 °C and the trichloroacetic acid (TCA)-insoluble radioactivity determined as follows: aliquots were transferred to strips of Whatman No. 1 paper, heated to 90 °C in 10% TCA containing 0.1% (w/v) unlabelled methionine (10 ml per strip) for 20 min, washed with cold 10% TCA (20 ml per strip), followed by ethanol and ether. The dry strips were counted in 4 ml scintillant (0.5% PPO, 0.03% POPOP in toluene). The remaining assay incubations were prepared for gel electrophoresis by addition of sodium dodecyl sulphate (SDS) and 2-mercaptoethanol to final concentrations of 1% (w/v) and 2% (v/v), respectively, and 1/20 volume of 20×NURB (Neville upper reservoir buffer²⁷). The samples were heated to 95 °C for 2 min and then analysed on slab polyacrylamide gels as described by Laemmli²⁸, using a resolving gel which contained a linear gradient of 7.5–25% (w/v) acrylamide. Electrophoresis was for 16 h at 14 mA at room temperature; the gels were stained in 0.1% (w/v) Coomassie brilliant blue R in 45% methanol, 10% acetic acid and destained in successive changes of 45% methanol, 10% acetic acid. The gels were impregnated with PPO as described²⁹, and the sensitivity of the film increased by pre-flashing³⁰. The film was scanned by a Joyce-Loebl densitometer. The traces are of the products of poly A-RNA isolated from pea leaves greened for a, 24 h; b, 12 h. The arrow marks the band which increases relative to the other bands. Each gel track contains the same amount of TCA-insoluble radioactivity (150,000 c.p.m.); this amount of radioactivity was directed by 0.5 µg added RNA. Controls containing no added RNA incorporated 2,500 c.p.m. The following molecular weight markers were used: RBPCase large subunit (55,000); soybean trypsin inhibitor (25,000); myoglobin (18,000); cytochrome c (12,000).

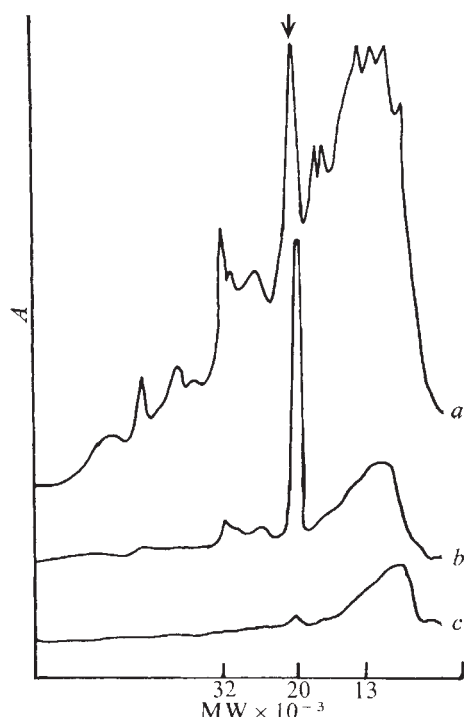


Fig. 2 Identification of small subunit precursor by immunoprecipitation. The ^{35}S -labelled products of translation of poly A-RNA in the wheat-germ system were prepared as described in Fig. 1. After 60 min at 27°C , the incubations were made up to 100 μl by addition of phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) and Nonidet P40 to final concentrations of $100\text{ }\mu\text{g ml}^{-1}$ and 1% (v/v) respectively. To each incubation 100 μg of rabbit immunoglobulin G (IgG) was added, and the mixture held at 37°C for 60 min. The specific anti-RBPCase IgG was prepared as described by Shapiro *et al.*³¹, using antigen affinity columns and DEAE/CM-cellulose chromatography; the IgG from pre-immune serum was isolated by ammonium sulphate precipitation followed by the ion-exchange chromatography step. Antigen-antibody complexes were precipitated by addition of excess (10 mg) of heat-inactivated mixture were taken and kept on ice whilst chloroplasts were isolated as described previously¹¹. Chloroplasts from 15 g of 11-d-old pea leaves were washed once in isolation medium and resuspended in 6 ml of 25 mM HEPES-KOH, 110 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol, pH 7.6. Aliquots (20 μl) of this suspension were added to the labelled products and incubated at 27°C for the times stated; controls contained 20 μl resuspension buffer. Chlorophyll was measured by the procedure of Arnon³³. *Staphylococcus aureus* (NCTC 8530)³². The *S. aureus* precipitates were washed twice with 250 μl of PBS containing BSA and Nonidet P40. The bound material was released by incubating the *S. aureus* at 50°C in 200 μl of 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1% SDS, 1% 2-mercaptoethanol. The bacteria were removed by centrifugation and the supernatants prepared for polyacrylamide gel electrophoresis and analysed as described in Fig. 1. The traces are as follows: *a*, total poly A-RNA-directed products, untreated; *b*, total poly A-RNA-directed products precipitated by anti-RBPCase IgG and *S. aureus*; *c*, as (*b*) but with preimmune serum instead of anti-RBPCase. The arrow indicates the small subunit precursor of apparent MW 20,000.

Processing of P20 to small subunit by intact isolated chloroplasts

Incorporation of ^{35}S -methionine into P20 by wheat-germ ribosomes programmed with pea poly A-RNA ceases after 60 min of incubation at 27°C . When wheat-germ extract containing labelled P20 is subsequently incubated with preparations containing intact pea chloroplasts, there is a reduction in the amount of labelled P20 and an appearance of label in the small subunit region of the polyacrylamide gel (Fig. 3). The identity of the small subunit labelled by this procedure was confirmed by immunoprecipitation with antiserum to RBPCase (Fig. 4). When analysed by the two-dimensional method of O'Farrell²³, this processing is seen to introduce label into both the electrofocusing variants of the small subunit polypeptide that are found

in *Pisum sativum*. Two observations suggest that this processing step does not require concomitant protein synthesis by either cytoplasmic or chloroplast ribosomes. First, processing occurs after protein synthesis by the wheat-germ extract has ceased, as judged by amino acid incorporation measurements. And second, processing still occurs in the presence of either chloramphenicol ($100\text{ }\mu\text{g ml}^{-1}$) or cycloheximide ($100\text{ }\mu\text{g ml}^{-1}$). Chloramphenicol is a specific inhibitor of protein synthesis by chloroplast ribosomes, while cycloheximide is a specific inhibitor of protein synthesis by cytoplasmic ribosomes¹. While it is clear that processing does not require concomitant protein synthesis, it is still possible that it requires the presence of ribosomes. This possibility has been ruled out by our finding that processing still occurs even if ribosomes have been removed from the wheat-germ extract by centrifugation (data not shown).

Processing of P20 to small subunit occurs maximally in the presence of chloroplasts which retain their outer envelope (Table 1). Lysed chloroplasts and stromal preparations show some activity (40% and 25%, respectively, of the activity of an equivalent amount of intact chloroplasts). The preparation of lysed chloroplasts is unfractionated and thus still contains fragments of envelopes. The slight processing activity of the stromal fraction can be removed by centrifugation at $30,000g_{av}$ (average) for 40 min. Preparations of chloroplast lamellae and heated chloroplasts (2 min at 90°C) have no processing activity.

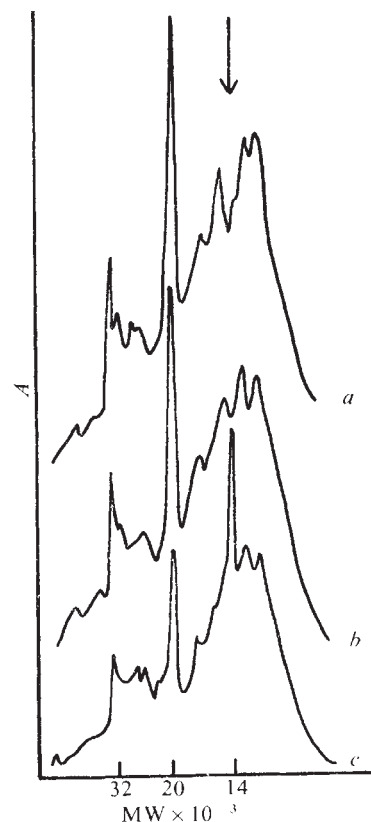


Fig. 3 Processing of P20 to small subunit by intact chloroplasts. Poly A-RNA was translated in the wheat-germ system as described in Fig. 1. After 60 min 10 μl aliquots of the incubation mixture were taken and kept on ice whilst chloroplasts were isolated as described previously¹¹. Chloroplasts from 15 g of 11-d-old pea leaves were washed once in isolation medium and resuspended in 6 ml of 25 mM HEPES-KOH, 110 mM KCl, 3 mM MgCl_2 , 10 mM dithiothreitol, pH 7.6. Aliquots (20 μl) of this suspension were added to the labelled products and incubated at 27°C for the times stated; controls contained 20 μl resuspension buffer. Chlorophyll was measured by the procedure of Arnon³³. Samples were prepared for polyacrylamide gel electrophoresis and analysed as described in Fig. 1. The traces are densitometer scans. Trace *a*, *in vitro* product incubated with buffer for 60 min; *b*, *in vitro* product incubated with chloroplasts (5 μg chlorophyll) for 0 min; *c*, *in vitro* product incubated with chloroplasts for 60 min. The arrow indicates the position of the small subunit.

These observations suggest that the processing enzyme is located in the chloroplast envelope.

The small subunit precursor reported from *Chlamydomonas*²² was also found to be processed to the small subunit, but in this case the processing activity was soluble. It is not possible to isolate intact chloroplasts from this alga; instead, extracts are prepared by the relatively violent method of passage through a French pressure cell. We therefore suggest that the soluble nature of the processing activity in this case may be a result of the procedures used, and not reflect its true location in the chloroplast envelope.

Transport of P20 into isolated chloroplasts

Our conclusion that the processing of P20 to small subunit requires the presence of chloroplast envelopes led us to see whether the small subunit would enter into the intact chloroplast. The commonly accepted criterion for the passage of a polypeptide across a membrane is the appearance of resistance to digestion by an added protease; this resistance should be abolished by the addition of detergents which disrupt membrane structure²⁴. Figure 5 shows the results of experiments designed to apply this criterion to the processing of P20 by intact chloroplasts. The results clearly show that the small subunit produced when labelled P20 is incubated with intact chloroplasts is resistant to hydrolysis by trypsin (track *c*), unless detergent is added at the same time (track *d*). Other low molecular weight polypeptides synthesised from poly A-RNA by the wheat-germ extract are all digested by trypsin, even in the absence of detergent. The product with the mobility of small subunit seen in track *a* was shown not to be authentic small subunit by tryptic peptide analysis (data not shown).

Mechanism for polypeptide transport into chloroplasts

The signal hypothesis provides an attractive mechanism for explaining how polypeptides which are secreted by cells are

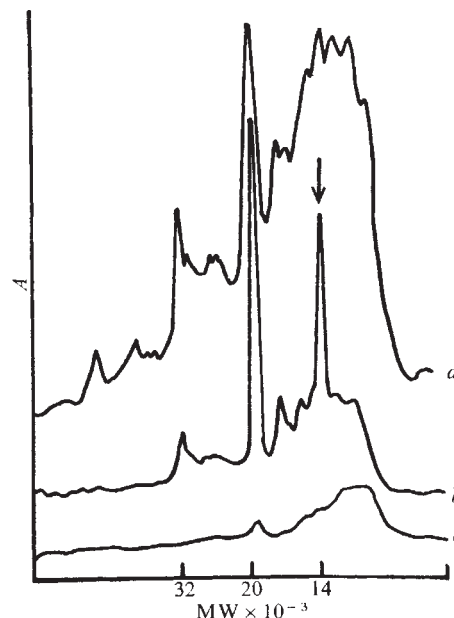


Fig. 4 Identification of product of *in vitro* processing of P20. Poly A-RNA was translated in the wheat-germ system and the labelled polypeptides obtained incubated with intact chloroplasts as described in Fig. 3 except that 10 μ l of wheat-germ incubation mixture was incubated in a final volume of 60 μ l with chloroplasts containing 6.8 μ g chlorophyll. After 60 min at 27 °C the chloroplasts were lysed by addition of Nonidet P40 to 1% (w/v) and the volume made up to 200 μ l with PBS. Aliquots (100 μ l) were analysed by immune precipitation and gel electrophoresis as described in Fig. 2. Electrophoresis was from left to right and autoradiography was for 7 d at -70 °C. The lines are densitometer scans. *a*, Total poly A-RNA-directed products incubated with intact chloroplasts; *b*, the polypeptides from *a*, which bind to anti-RBPCase IgG; *c*, as (*b*) but using pre-immune IgG. The arrow marks the position of RBPCase small subunit.

Table 1 Processing of P20 by chloroplast fractions

Incubation for 60 min in the presence of:—	Processing activity
Buffer	0
Intact chloroplasts	100
Lysed chloroplasts	40
Low speed stroma	25
High speed stroma	< 5
Thylakoids	0
Boiled chloroplasts	0

Poly A-RNA was translated in the wheat-germ system as described in Fig. 1. After 60 min, 10 μ l aliquots of the incubations were taken and kept on ice whilst chloroplasts were isolated as described in Fig. 3. Two pellets of chloroplasts were obtained. One was resuspended in 6 ml of 25 mM HEPES-KOH, 110 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, pH 7.6 (intact plastid buffer); this preparation is referred to as intact chloroplasts. The other pellet was resuspended in 3 ml of 10 mM HEPES-KOH, 10 mM dithiothreitol, pH 7.6 followed by 3 ml of 2 $\times$ intact plastid buffer; in this preparation all the chloroplasts are lysed. An aliquot (1 ml) of the intact chloroplast suspension was placed, for 5 min, in a boiling waterbath; this gave a boiled chloroplast preparation. An aliquot (4 ml) of the lysed chloroplast preparation was centrifuged at 4,000 *g* for 5 min, the supernatant was removed and the pellet resuspended in 4 ml of intact plastid buffer. The two fractions are referred to as low speed stroma and thylakoid preparations respectively. Half of the low speed stroma was centrifuged for 40 min at 30,000 *g*_{av}; this gave high speed stromal preparation. Equal volumes of each preparation were added to the labelled products and incubated, in a final volume of 50 μ l, at 27 °C for the times stated. Chlorophyll was measured by the procedure of Arnon²⁵; each incubation contained the equivalent of 4.7 μ g of chlorophyll. Samples were prepared for polyacrylamide gel electrophoresis and analysed as described in Fig. 1. The autoradiographs were scanned on a Joyce-Loebl microdensitometer. The processing activity was determined by comparing the absorbance of the P20 band at 0 and 60 min. Incubation with intact chloroplasts caused a 60% reduction in this absorbance; this decrease was arbitrarily defined as 100 units of activity.

synthesised by membrane-bound ribosomes and transported into the lumen of the endoplasmic reticulum^{16,17}. According to this hypothesis, secreted polypeptides are encoded by messengers which specify an N-terminal sequence of amino acids, whose appearance on the polysome causes the latter to bind to the membrane of the endoplasmic reticulum. The growing polypeptide chain is then inserted through a tunnel in the membrane formed by the association of the signal sequence with membrane proteins; protease activity in the membrane rapidly removes the signal sequence. A key feature of this mechanism is that the transport through the membrane depends upon concomitant protein synthesis by membrane-bound ribosomes, since the polypeptide chain moves through the tunnel in an extended form as it is being lengthened.

Application of this hypothesis to the transport of polypeptides into chloroplasts predicts that the outer chloroplast envelope should be studded with bound ribosomes during the development of the organelle. However, it is apparent from many electron microscopic studies that the chloroplast envelope never presents a similar appearance to rough endoplasmic reticulum. To overcome this objection, a modified signal hypothesis may be considered, in which the chloroplast polypeptides are first inserted into vesicles of endoplasmic reticulum which subsequently fuse with the chloroplasts.

It is clear from the evidence we have presented that the transport of the small subunit of RBPCase into the chloroplast proceeds by a mechanism different from that envisaged by the signal hypothesis. This transport proceeds after synthesis of the precursor has ceased, and in the absence of cytoplasmic ribosomes. Additional evidence consistent with this conclusion is the observation that the small subunit of *Chlamydomonas* is synthesised by free cytoplasmic ribosomes in the absence of membranes²². All these observations are consistent with the

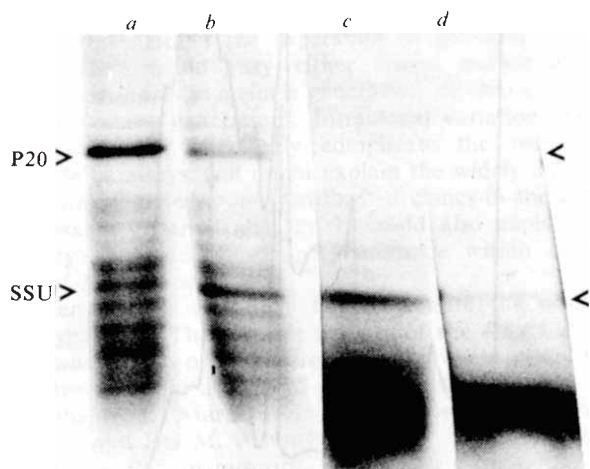
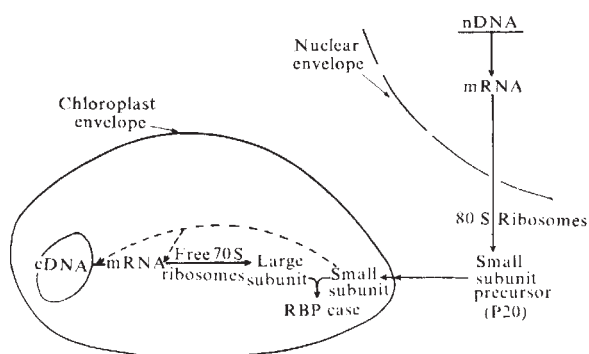


Fig. 5 Transport and processing of P20 by intact chloroplasts. Poly A-RNA was translated in the wheat-germ system and the labelled polypeptides obtained were incubated with intact chloroplasts as described in Fig. 4. At the end of the incubation, TPCK-treated trypsin was added to a final concentration of $100 \mu\text{g ml}^{-1}$ and the mixture incubated at 30°C for 30 min. To some samples Nonidet P40 (final concentration 1% (v/v) was added at the same time as the trypsin. Samples were analysed as in Fig. 2. Track *a*, poly A-RNA-directed products incubated in buffer without chloroplasts for 60 min followed by a further 15 min in the absence of trypsin; *b*, poly A-RNA-directed products incubated with chloroplasts for 60 min followed by 15 min in the absence of trypsin; *c*, as (*b*), but the final incubation was with trypsin; *d*, as (*c*), but the final incubation was with Nonidet P40 as well as trypsin. P20, small subunit precursor; SSU, small subunit of RBPCase.

envelope carrier hypothesis which was first suggested to account for the transport of polypeptides into chloroplasts when it was demonstrated that isolated chloroplasts synthesise the large but not the small subunit of the RBPCase¹¹. This hypothesis states that a class of proteins exists in the chloroplast envelope which recognises sites common to all those proteins which are made on cytoplasmic ribosomes, but which are destined to function in the chloroplast. In this hypothesis the recognition site on the entering polypeptide need not be at the N-terminus.

The weakness of the envelope carrier hypothesis is that while it accounts for the specificity of transport, it does not propose a mechanism for the actual movement of the polypeptide through the envelope. However, analysis of the processing of P20 on electrofocusing gels shows that P20 has a much lower isoelectric point (by 1.0–1.5 pH units) than either of the two electrofocusing variants of the mature small subunit. This observation suggests that the extra sequence(s) in P20 contain acidic amino acids; known signal sequences, by contrast, are rich in hydrophobic amino acids and contain few, if any, acidic amino acids³⁴.

Fig. 6 Postulated model for RBPCase synthesis in higher plants. nDNA and cDNA stand for nuclear and chloroplast DNA respectively. The dashed line indicates possible control sites at which small subunit affects synthesis of large subunit (modified from ref. 5).



Removal from P20 of such a large number of amino acids (around 50), many of which are charged, is likely to cause a conformational change in the polypeptide. We therefore propose that the transport of P20 into chloroplasts involves combination with a specific carrier in the chloroplast envelope. Protease activity in the envelope then removes part of the polypeptide, which triggers a conformational change leading to the transport of the small subunit across the envelope and release into the stroma.

Aspects of the envelope carrier hypothesis are testable by established techniques. Purified preparations of pea chloroplast envelope can be made³⁵, and will be tested for combination with and processing of P20. The physical state of the small subunit inserted into both intact chloroplasts and purified envelopes will be determined to see whether combination with the large subunit is necessary for the release of the small subunit into the stroma. It is possible that part of the sequence difference between P20 and the small subunit is necessary for the assembly of the RBPCase molecule: this would account for the difficulty in reconstituting active RBPCase molecules from isolated subunits. The suggestion that the small subunit is required for the continuing translation of the messenger for the large subunit⁵ can also now be tested.

Figure 6 summarises our current model for the synthesis of RBPCase in eukaryotic plant cells. The principles exemplified by this model may be of general application to the synthesis of the many proteins found in both chloroplasts and mitochondria. It has been established for each of at least three mitochondrial proteins that their subunits are made in different cellular compartments, for example, three of the seven subunits of cytochrome *c* oxidase are synthesised by mitochondrial ribosomes while the other four are made in the cytoplasm³⁵. The cytoplasmic subunits have been reported to exert a specific and positive effect on the synthesis of the other three subunits by isolated mitochondria in the absence of cytoplasmic protein synthesis³⁶. This finding suggests that in the case of mitochondria as well as chloroplasts, cytoplasmically synthesised polypeptides enter the organelle by an envelope carrier type of mechanism, and control the synthesis of the polypeptides with which they ultimately associate.

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letters to nature

Cosmic ray source abundance of calcium

MEASUREMENTS of the charge composition of cosmic ray nuclei have led to the belief that cosmic ray calcium nuclei originate in about equal proportions from the sources and from spallation in the interstellar medium¹. The primary calcium is thought to be essentially ⁴⁰Ca, as in the Solar System, while from nuclear reaction systematics the spallative calcium should be mainly ⁴²Ca, ⁴³Ca and ⁴⁴Ca. Simpson *et al.*², however, have presented some surprising results of a measurement of the isotopic composition of calcium in cosmic rays. They found that calcium is largely ⁴²Ca, and they did not see any ⁴⁰Ca. They concluded that calcium is absent at the source, and to account by spallation only for the measured abundances they assumed that the cross-sections for production of Ca by spallation of Fe are much larger than those calculated by Silberberg and Tsao's formula³. A very low calcium abundance in the source would be a major difference with the Solar System, and an experimental determination of the calcium production cross-sections is essential to settle this point. In a report on results of measurements of cross-sections for production of stable nuclides by spallation of Fe (ref. 4) we emphasised the element group Sc–Mn, but some results have also been obtained for Ca, as a byproduct⁵. We considered these as preliminary, and no cross-section value was given for this element. The measurement of Simpson *et al.*² has motivated us to re-examine our calcium data. The cross-sections we

Although the experiment was not specially designed for calcium, this problem was not so crucial here, because of the large difference in isotopic composition between the spallative calcium and the natural one. Contamination is dominant only for ⁴⁰Ca, which represents 97% of natural calcium, but this is not important because the cross-section of ⁴⁰Ca is very small¹. On the other hand, isotopes with larger cross-sections have very low natural abundances, so that the contamination, as deduced from the mass-40 peak, is only a few per cent for ⁴²Ca and ⁴³Ca, and at most ~20% for ⁴⁴Ca.

In Table 1, we give the measured isotopic ratios, corrected for contamination, and isotopic discrimination of the mass-spectrometer. Two determinations have been performed at 600 MeV, and one at 21 GeV. The first three ratios give the relative composition of the four isotopes with the largest cross-sections. These are the most precisely measured. As we need the radioactive isotope ⁴⁵Ca (*t*_{1/2} = 165 d) for deriving absolute cross-sections, we also give two ratios relative to this radioisotope. The larger uncertainties in this case are due to both the smaller cross-sections of ⁴⁵Ca and also to the scandium contribution to the mass-45 peak.

Because ⁴⁵Ca is pure β^- -emitter, measurements of the cross-sections are difficult and few have been reported; 1.20 $\pm$ 0.13 mb is given at 660 MeV, 1.4 $\pm$ 0.2 mb at 730 MeV, and 0.69 $\pm$ 0.14 mb at 24 GeV (refs 6, 7, 8 respectively). We do not know of a more recent determination. The good agreement between the values at 660 and 730 MeV gives

Table 1 Ratios of cross-sections for calcium production by spallation of iron, measured by mass-spectrometry

	⁴¹ Ca/ ⁴² Ca	⁴³ Ca/ ⁴² Ca	⁴⁴ Ca/ ⁴² Ca	⁴² Ca/ ⁴⁵ Ca	⁴⁶ Ca/ ⁴⁵ Ca
600 MeV	0.36 $\pm$ 0.05	1.18 $\pm$ 0.11	1.24 $\pm$ 0.14	9.1 $\pm$ 2.0	0.27 $\pm$ 0.07
21 GeV	0.42 $\pm$ 0.06	1.08 $\pm$ 0.10	1.10 $\pm$ 0.15	9.5 $\pm$ 2.8	0.23 $\pm$ 0.07

have derived and reported here, although not of the precision we would like, allow a safe enough conclusion on the Ca source abundance.

The experimental procedure has been described elsewhere^{4,5}. We recall that ultra-high purity iron targets were irradiated by protons from the two CERN accelerators (600 MeV and 21 GeV); the spallation products were then chemically separated, and their isotopic composition determined by mass spectrometry. The cross-sections of stable isotopes were obtained with respect to those of radioactive isotopes, measured by classical nuclear physics techniques.

One of the major problems in measuring small quantities of stable isotopes is the contamination by natural impurities.

us some confidence but a new measurement, at least at high energy, would be desirable. Combining these values with our measured isotopic ratios, we derive the absolute cross-sections given in Table 2. These are cumulative cross-sections—including the contributions from short-lived isobars.

The data of Simpson *et al.*² were obtained at ~ 500 MeV m⁻¹. Taking into account solar modulation and interstellar energy loss, the relevant cross-sections should be taken, typically, around 1 GeV. On the basis of known excitation functions, cross-sections at this latter energy are expected to be of the same order as, or slightly larger than those at 600 MeV. Our values are considerably smaller than those adopted by Simpson *et al.*² (70 mb for total Ca,

Table 2 Experimental cross-sections (mb) for calcium production by spallation of iron, compared with Silberberg and Tsao's calculations

	⁴¹ Ca	⁴² Ca	⁴³ Ca	⁴⁴ Ca	⁴⁵ Ca	⁴⁶ Ca
600 MeV	3.9 $\pm$ 1.2	10.9 $\pm$ 2.7	12.9 $\pm$ 3.1	13.5 $\pm$ 3.0	1.20 $\pm$ 0.13*	0.32 $\pm$ 0.09
600 MeV calc.†	2.16	11.82	11.60	12.11	1.23	0.39
21 GeV	2.8 $\pm$ 1.2	6.6 $\pm$ 2.3	7.1 $\pm$ 2.5	7.2 $\pm$ 2.5	0.69 $\pm$ 0.14*	0.16 $\pm$ 0.06
> 2.3 GeV calc.†	3.11	15.68	13.98	13.20	1.24	0.35

*Normalisation, ref. 6 (660 MeV), ref. 7 (24 GeV).

†Ref. 3.

27 mb for ^{42}Ca . At 600 MeV, they agree quite well with those calculated by Silberberg and Tsao's formula³, also given in Table 2. The large discrepancy at high energy is essentially due to our normalising to the ^{42}Ca cross-section measured by Estrup⁸, which is almost a factor of 2 smaller than the calculated one; the disagreement on isotopic ratios is much smaller.

An independent upper limit of the ^{42}Ca cross-sections can be deduced from our scandium measurements⁴. At 600 MeV we made six determinations of the spallation isotopic ratio $^{42}\text{Sc}/^{46}\text{Sc}$, and four at 21 GeV, at times after irradiation varying from 1 d to 280 d (when about 70% of ^{42}Ca had decayed to ^{42}Sc). We observed no systematic increase of this ratio with time, and all values agreed within 2% at 600 MeV, and 5% at 21 GeV, well within experimental uncertainty. This yields conservative upper limits of the ^{42}Ca cross-sections of ~ 1.7 mb at 600 MeV, consistent with the measured value⁸, and ~ 1.8 mb at 21 GeV. The derived upper limits at high energy for ^{42}Ca and total Ca are thus still lower than Simpson *et al.*'s assumption².

We conclude, therefore, that in spite of the rather large uncertainties of our results, it is very unlikely that the observed cosmic ray calcium abundance can be entirely due to interstellar spallation. Moreover, in secondary calcium, ^{42}Ca , ^{43}Ca and ^{44}Ca should have about the same abundance. Thus, the previous assumption that calcium is present in the source with an abundance ~ 10 –15% that of iron is probably correct, but if one accepts the data of Simpson *et al.*² the source calcium must be mainly ^{42}Ca . This would be an enormous difference with Solar System abundances. In addition, measurement of the isotopic composition of other primary cosmic ray species^{2,9–11} show that they are all consistent with Solar System composition, although there are claims for some enhancement of neutron-rich isotopes of Ne (ref. 9) and Fe (ref. 2). Thus, one would have to find a process of nucleosynthesis which would not only make more ^{42}Ca than ^{40}Ca but at the same time leave all other elements with 'normal' composition. This seems to be quite difficult and considering the low statistics of the calcium measurement of Simpson *et al.*², it seems reasonable to wait for further confirmation of the strange isotopic composition of cosmic ray calcium.

The experimental work was carried out while I was with Laboratoire René Bernas, Orsay, France.

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Interpretation of observed cosmic microwave background radiation

ALFVEN and Mendis¹ concluded recently that dust grains in galaxies rendered the Universe opaque to the cosmic microwave background at a red shift of $z = 40$, instead of the generally accepted value of $z = 1,500$. This is highly significant because the microwave background is usually believed to be evidence for a

very hot, dense phase of the Universe². If we can see no further back than $z = 40$, then one objection to nonstandard³ (no big bang) cosmological models is removed. I present here some arguments to show that their assumptions are unreasonable and that a proper calculation in the standard big bang model shows that dust grains are transparent to the microwave background.

Alfven and Mendis¹ calculated the opacity of galactic dust grains by integrating over the metagalaxy at one epoch. A proper treatment would integrate back in time from the present epoch. This alone reduces their estimate of the opacity by a factor of ~ 5 . They also assumed no evolutionary effects in their calculations. But the dust grains are thought⁴ to consist of CH_4 , NH_3 and H_2O with silicate or graphite cores. The mantles sublime⁴ at 100 K, which was the temperature at epoch $z = 36$. The remaining cores have an absorption cross-section roughly 10 times lower⁵ than the ice-coated grains. Another important evolutionary effect concerns the nucleosynthesis of the elements that make up the grains. In the standard big bang model, elements as heavy as oxygen and silicon could not be synthesised in the big bang itself⁶. They must have been synthesised in stars, most likely at an epoch no earlier than $z = 65$. Thus dust grains could not have existed in significant amounts before this era. This will be discussed in more detail later.

Alfven and Mendis¹ calculated the opacity for 1.8 mm microwaves, which they used as the representative wavelength of the cosmic black body radiation. Galactic dust grains, however, are more transparent to longer wavelengths. Observations of the radiation have been made at varying wavelengths⁷ up to 73.5 cm, for which the expected opacity of the grains is less by a factor of 400.

The value of the deceleration parameter q_0 is not well known. Alfven and Mendis used $q_0 = 0.03$. If $q_0 = 0.5$ (flat space), then the opacity of dust grains is reduced by a factor of 4 for a given epoch z . This is because a large deceleration means that the cosmic expansion was faster in the past, so that a ray of light spent less time in any given epoch. A value of 0.5 for q_0 does not contradict any data.

The optical depth τ , for a given wavelength λ_0 along a path from the present era to a past epoch of red shift z_e , is given by

$$\tau(\lambda_0) = \int_0^{z_e} n(z)\sigma(\lambda)dz \quad (1)$$

where σ is the scattering or absorption cross-section per grain, and n is the number density of the grains. The number of dust grains in a comoving volume remains constant, except for evolutionary changes. As the volume increases as $(z+1)^{-3}$, the number density increases as

$$n(z) = (z+1)^3 f(z) n(0) \quad (2)$$

where $f(z)$ contains all evolutionary effects such as the nucleosynthesis of elements that make up the grains, and is normalised to $f(0) = 1$.

In a matter-dominated Friedmann universe (standard big bang model), a straightforward but messy calculation shows that we can express $d\tau$ as

$$d\tau = -cdt = (c/H_0)(z+1)^{-2}(1+2q_0z)^{-1/2} dz \quad (3)$$

Starting with a measurement of $\sigma(\lambda)$ at $\lambda = 0.55 \mu\text{m}$, Alfven and Mendis¹ assumed that $\sigma(\lambda) \propto 1/\lambda$ and extrapolated $\sigma(\lambda)$ to $\lambda_0 = 1.8 \text{ mm}$. Detailed models of the grains⁵ support this functional dependence in a rough way. The evaporation of the grain mantles, however, requires that the opacity drops sharply when the temperature rises above 100 K. This occurred for all epochs

before $z = 36$. As $\lambda \propto R \propto (z+1)^{-1}$ where R is the cosmic scale factor, we can write

$$\sigma(\lambda) = (z+1)g(z)\sigma(\lambda_0) \quad (4)$$

where

$$g(z) = \begin{cases} 1.0 & z < 36 \\ 0.1 & z > 36 \end{cases} \quad (5)$$

Combining equations (1), (2), (3) and (4) we obtain

$$\tau(\lambda_0) = (c/H_0)n(0)\sigma(\lambda_0) \int_0^{z_c} (z+1)^2 f(z) g(z) (1+2q_0 z)^{-1/2} dz \quad (6)$$

At optical wavelengths, the optical depth of our Galaxy parallel to its polar axis is roughly⁸ 0.5. Let D , S_g and n_g be the thickness, area and dust density of the dusty region of our Galaxy. Then

$$Dn_g\sigma(0.55 \mu\text{m}) = 0.5 \quad (7)$$

and

$$n(0) = DN_{0g}S_g n_g \quad (8)$$

where N_{0g} is the number density of galaxies. Alfven and Mendis used values of $S_g = 5 \times 10^{45} \text{ cm}^2$ and $N_{0g} = 1.2 \times 10^{-75} \text{ cm}^{-3}$, both of which are reasonable estimates. Combining these values with equations (7), (8) and $\sigma(\lambda) \propto 1/\lambda$ gives

$$n(0)\sigma(\lambda_0) = 1.65 \times 10^{-33}/\lambda_0(\text{mm}) \text{ (in cm}^{-1}\text{)}. \quad (9)$$

For⁹ $H_0 = 55 \text{ km (s-Mpc)}^{-1}$,

$$c/H_0 = 1.6 \times 10^{28} \text{ cm}, \quad (10)$$

which is the same value Alfven and Mendis used for R_0 .

The only thing needed to finish the calculation of the opacity of the galactic dust grains is the evolution of dust grain density, represented by the function $f(z)$. As dust grains are composed mostly of heavy elements, restrictions on the heavy element abundances will restrict grain densities. Observations of external galaxies by Strom *et al.*¹⁰ show gradients of metal abundances, with the outer regions containing fewer heavy elements than the nuclei. This can be explained¹¹ only by assuming that stellar formation took place during galaxy formation, not before. As stars formed in a predominantly gaseous infall, their nucleosynthesis and subsequent deaths as supernovae (or their stellar winds) enriched the galactic cores more so than the outer regions. I assume that before the Galaxy formed, there was some initial abundance of heavy elements from pregalactic stars. To estimate this abundance, I use the observed heavy element abundance of the poorest metal containing globular clusters, since they are thought to be the oldest objects that can be seen¹². Their metal abundances are roughly 1/300 that of the Sun¹², so $f(z > z_g) = 0.003$, where z_g is the epoch of Galaxy formation. If we assume that our Galaxy started as a spherical cloud that collapsed to a disc, the observed mass and radius of the Galaxy gives a free fall time of roughly $(r^3/GM)^{1/2} \simeq 100 \text{ Myr}$. As the epoch $z = 65$ occurred about 100 Myr after the big bang, the galaxies couldn't form earlier than the epoch $z = 65$. Thus an upper bound for $f(z)$ is

$$f(z) = \begin{cases} 1.0 & z < 65 \\ 0.003 & z > 65 \end{cases} \quad (11)$$

Combining equations (5), (6), (9), (10) and (11) enables us to do the integral and calculate $\tau(\lambda_0)$. Recent measurements¹³ of the black body anisotropy used a wavelength of $\lambda_0 = 9 \text{ mm}$. At

$z_c = 1,500$, the generally accepted value of decoupling, the optical depth due to absorption by galactic grains is $\tau(9 \text{ mm}) = 0.18$ for $q_0 = 0.03$ and $\tau(9 \text{ mm}) = 0.05$ for $q_0 = 0.5$. Thus most of the light emitted at the time of decoupling has reached us unscattered by intervening dust.

To conclude, I have calculated the opacity of galactic dust grains to the microwave background radiation from the time of decoupling at $z_c = 1,500$ to the present in the standard big bang model. I have estimated evolutionary effects on grain opacity and abundance, conservatively overestimating the expected grain density. At typical wavelengths used in studying the microwave background, I have shown that the optical depth of the grains is only 0.18 for $q_0 = 0.03$ and is 0.05 for $q_0 = 0.5$. Thus the microwave background can give us information on an early dense phase of the Universe.

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Plasma fluxes between ionosphere and protonosphere

PLASMA flux between ionosphere and protonosphere is important for our understanding of ionospheric–magnetospheric coupling. Attempts have been made to deduce the H^+ flux between ionosphere and protonosphere from incoherent scatter observations of O^+ densities and vertical velocities in the topside ionosphere^{1–4}, but theoretical studies⁵ have suggested that the O^+ flux is not necessarily related in a simple way to the H^+ flux and may even at times be in the opposite direction. More direct estimates of average daytime and night-time fluxes have been calculated from a limited data base of whistler observations of tube content⁶, while topside electron density profiles have also been used⁷.

The ATS-6 radio beacon experiment⁸ provides a method of estimating integrated net ionospheric–protonospheric fluxes on a continuous basis from the temporal changes in protonospheric content. The receiving station at Aberystwyth (52.42°N, 4.05°W) for transmissions from the ATS-6 satellite (geostationary above 35°E) allowed determination of both the plasmaspheric group delay and the ionospheric polarisation rotation. The former is related directly to the total electron content (N_T) along the slant path from satellite to receiver while the latter gives a measure of the Faraday electron content (N_F) along the ionospheric part of the path.

Model studies^{15–17} have shown that using an average longitudinal gyrofrequency for the path, evaluated at a fixed height in the range 350–420 km, in the conversion of polarisation rotation to Faraday content produces values which give a measure of the electron content up to an effectively constant altitude between 2,000 and 3,000 km. For geometry applicable to the present study the height sensitivity of the longitudinal

gyrofrequency is small, differing, over the height range 300 to 500 km, by less than $\pm 1.3\%$ from the chosen value for 420 km.

From the difference between N_T and N_F measurements made to a sufficient degree of accuracy, the electron content of the protonospheric part of the path (N_p) can be obtained. In practice, N_p is a measure of the content from $\sim 2,500$ km altitude to the plasmapause, estimated to an absolute accuracy within $\pm 15\%$, but with much better relative accuracy allowing small temporal changes to be studied. The protonospheric content expressed as a percentage of the total ranges in general from some 15–20% by day to 30–40% by night.

Temporal changes of protonospheric content are indicative of the filling and draining of this region where production and loss processes are unimportant so that the ionospheric–protonospheric flux can be obtained from the temporal gradient of the protonospheric content (dN_p/dt). It should be noted that the protonospheric content measured by the ATS-6 experiment refers to a slant path to the satellite which intersects a wide range of L -value flux tubes, as shown in Fig. 1 for Aberystwyth geometry up to $L = 4$. The protonospheric flux estimated from ATS-6 measurements is thus an integrated parameter over these tubes in contrast to the more direct measurements of flux, essentially along a particular field tube, given by the whistler and incoherent scatter techniques.

Monthly median diurnal values of N_p have been used to estimate the temporal rate of change of this parameter (dN_p/dt), calculated for each hour. To reduce the scatter in the data points, the N_p values were first filtered using a 3-h running mean before calculating the gradients. The diurnal variations of the fluxes for December 1975 and January 1976, representing northern hemisphere winter, and June and July 1976 corresponding to summer conditions are shown in Fig. 2. Positive values are indicative of an upwards flux filling the tubes with negative values corresponding to downwards depletion. The highly intermittent nature of the beacon transmissions, particularly while the satellite was eclipsing, have prevented reliable flux data being obtained for the equinoctial season.

To help explain some of the observed features of the diurnal variations of the integrated flux, the sunrise and sunset times, in both local (47°N , 22°E) and conjugate (35°S , 37°E) hemispheres at 350 km, at the ionospheric base of the $L = 2$ flux tube, which intersects the ATS-6 ray path at $\sim 5,000$ km altitude, are marked on Fig. 2.

In interpreting Fig. 2 it must be remembered that the satellite crosses many field lines at different heights, the ionospheric feet of which become sunlit at different times. Figure 1 indicates the range of latitudes of the ionospheric terminations of the field lines within the plasmasphere below $L = 4$, but taking account of the longitudinal range as well, the spread of sunrise and sunset times for the feet of the relevant field lines in both local and conjugate hemispheres can exceed ± 1 h about the times shown in Fig. 2. A second factor to consider is the time constant between an increase in ionospheric ionisation and the appearance of enhanced protonospheric plasma. A time constant ~ 1 h has been suggested for the diffusive barrier at the O^+/H^+ transition height⁹, while the travel time of ions

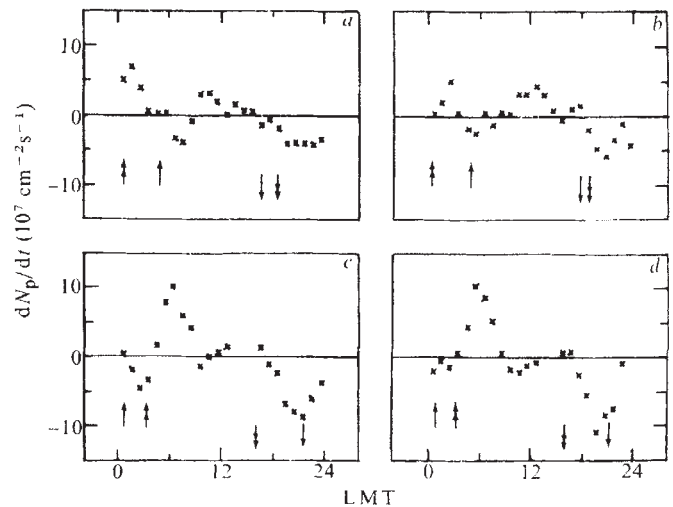


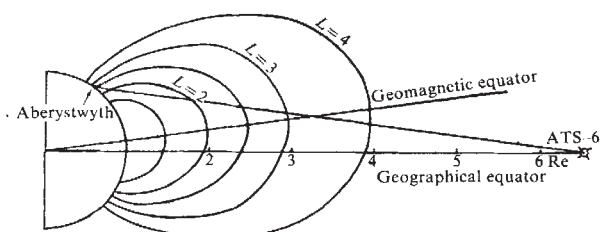
Fig. 2 Diurnal variations of the integrated protonospheric flux (dN_p/dt) for Aberystwyth for *a*, December 1975, *b*, January, *c*, June and *d*, July 1976. The arrows mark the times of sunrise and sunset in both local and conjugate hemispheres at the terminations at ionospheric height (350 km) of the $L = 2$ field line which intersects the ray path at $\sim 5,000$ km altitude. $\uparrow$, local sunrise; $\downarrow$, local sunset; $\uparrow$, conjugate sunrise; $\downarrow$, conjugate sunset.

to the protonosphere even at thermal speed may considerably exceed 1 h depending on the field line path. Thus the overall time constant may be of the order of several hours.

Considering first the Northern Hemisphere summer curves represented by June and July in Fig. 2, it can be seen that upwards fluxes start some 2 h after local sunrise with maximum values some 2 h after conjugate sunrise. The subsequent decrease in the flux rate may indicate that the relatively short small volume tubes of low L -value (say $L \leq 2$) become well filled so that the upwards flux to them is decreased and the subsequent small increase in protonospheric content occurs on the large volume higher L -value flux tubes¹⁰. The rates of change do not differ significantly from zero in the middle of the summer day when the neutral wind is depressing the F-layer ionisation, but after conjugate sunset a large downwards flux quickly develops. It is interesting that this downwards flux maximises at about the time of the second daytime ionisation peak in the still sunlit local ionosphere. This suggests that the protonospheric depletion at this time is to the conjugate ionosphere and the shape of the flux variation, quickly maximising downwards with subsequent smaller magnitudes, seems to indicate that the short inner tubes are quickly drained. The northern hemisphere winter curves represented by December and January show an upwards flux following sunrise in the local hemisphere and a sunset associated depletion. It is also interesting that a short lived upwards flux can be found around conjugate sunrise for these months when the time separation between sunrise in the two hemispheres is a maximum. This net upwards flux is of short duration, however, and the enhanced losses and effective downward movement of the local ionosphere evidenced by the well-known pre-dawn electron density depletion more than compensate for conjugate induced enhancements. A net decrease in protonospheric content is thus found until after local sunrise.

The average integrated fluxes obtained in the present study show that to within the accuracy of the estimates the upwards flux by day in magnitude and duration is broadly compensated by the downwards flux at night. Park⁶ from whistler observations has estimated average flux magnitudes generally greater than the levels indicated by the present study, with the average daytime upwards flux exceeding the downwards night-time flux by a factor of 2. While the present results are based on monthly median protonospheric content data, the values in

Fig. 1 Geomagnetic field line configuration for ATS-6 to Aberystwyth ray path.



Park's case study refer to a post-storm period during which the protonosphere is being replenished with daytime refilling exceeding night-time draining. Additionally, his observations are for tubes with $L > 4$ while the Aberystwyth ray path crosses tubes with $L \geq 1.7$ and it has been argued that the flux is enhanced for higher L -shells because of the relatively less dense plasma in these larger volume tubes¹⁰.

It can be concluded from this study that both local and conjugate ionospheres play important parts in the filling and depletion of the protonospheric flux tubes; a result supported by recent theoretical work¹¹. Thus in the interpretation of protonospheric content data corresponding to observations along the line of sight to a geostationary satellite, ionospheric-protonospheric interactions in both hemispheres must be considered. This conclusion enables us to account for the significant differences found between ATS-6 observations of protonospheric content made in Europe and USA. A set of protonospheric content data for July 1974 to May 1975 from ATS-6 observations made at Boulder, USA (40°N, 105°W) has been published¹². These monthly median values of N_p converted to local mean time show a daytime minimum in protonospheric content in winter and very low values in summer. By contrast, the Aberystwyth data (albeit for a later year but still near solar minimum) have a daytime maximum at all seasons with winter night-time magnitudes comparable to those at Boulder, but in summer the European content can be more than double that of the America station. A detailed examination¹³ shows that, in addition to the effects of observational sensitivities, these differences may be explained in terms of ionospheric-protonospheric interactions in both hemispheres, remembering that the nonalignment of the geographic and geomagnetic axes results in an ionosphere conjugate to Boulder which is sub-Antarctic in location (57°S, 105°W). The anomalous behaviour of the continuously sunlit Antarctic ionosphere in local summer (December-January) under the influence of the neutral wind is well documented¹⁴, and this factor, together with the long winter nights and the generally higher L -shells intersecting the Boulder ray path, may account for the differences between the European and American protonospheric contents.

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Post-geomagnetic storm protonospheric replenishment

THE response of the ionosphere to geomagnetic storms is well documented¹⁻³, although the complex interaction of the various physical mechanisms is not yet fully explained. However, the response of the overlying protonosphere to geomagnetic activity, a topic important to the understanding of ionospheric-magnetospheric coupling has not been widely studied. A direct study of tube content recovery obtained from whistler observations during an 8-d period in June 1965 following a sudden commencement storm has been published by Park⁴, although theoretical work⁵ has given a value for the replenishment time constant which is larger than that observed. We report here the results of protonospheric content observations during a period where a major sudden commencement storm was followed by an exceptionally long period of quiet allowing complete dynamic saturation of the protonospheric flux tubes to be reached.

The ATS-6 radio beacon experiment⁶ carried out at Aberystwyth (52.42°N, 4.05°W), while the geostationary satellite was stationed at 35°E longitude, allowed estimates to be made of both the plasmaspheric total electron content (N_T) and the ionospheric Faraday electron content (N_F) to sufficient accuracies that the protonospheric electron content along the slant path (N_p) could be obtained from the difference. We report here the results of protonospheric electron content measurements during the period 28 April to 21 May 1976. Hourly values of N_p available throughout the period are plotted in Fig. 1. Also shown for comparison, recurring for each day, is a plot of the diurnal variation of the averaged hourly N_p for May 1976. Values of the 3-h K_p index are presented above the corresponding N_p data. The period starts with 4 d in which the geomagnetic activity reaches $K_p=4$, late on 29 April and N_p shows a fluctuating pattern, particularly on 30 April and 1 May when the diurnal variation is almost masked by apparently random variations. A major storm with sudden commencement at 1828 UT on 2 May and K_p reaching 8, is preceded by a sharp increase in N_p around midday to be followed by a depletion with N_p reaching very low values on the morning of 3 May and even lower magnitudes around midnight of that day. In fact this value $1.3 \times 10^{16} \text{ m}^{-2}$ was the lowest N_p value found at Aberystwyth during the 9-month observing period. Three days follow with substantial breaks in the N_p data due to non-continuity of the satellite transmissions, but the values plotted show the trend of a replenishing protonospheric content which is continued until about 16 May during a time of quiet geomagnetic activity. Superposed on this trend is a regular diurnal variation with increasing amplitude as the quiet period continues. From 16 to 19 May the geomagnetic activity is very low and N_p is consistently above average with a continuing clear large diurnal variation. It therefore seems that the depleted protonosphere of 3 May requires some 14 d of quiet conditions with steady diurnal partial filling and draining to reach a quasi-equilibrium situation about 16 May. Thus from 16 to 18 May the protonospheric content for ATS-6 to Aberystwyth geometry presents its saturated dynamic equilibrium pattern for quiet geomagnetic activity with values maximising around $7 \times 10^{16} \text{ m}^{-2}$ during daytime filling and depleting to about $4 \times 10^{16} \text{ m}^{-2}$ at night. The maximum and minimum values of N_p for each day both increase steadily from the storm depletion with the daytime filling more than compensating the night-time draining for some 12-14 d. In the saturated dynamic equilibrium situation of 16-18 May the maximum daytime protonospheric electron content (N_p) makes up some 20% of the total electron content (N_T) as opposed to only 11% on the day following the sudden commencement.

The first point to note from this study of N_p changes is that the regular diurnal variation, with early morning minimum

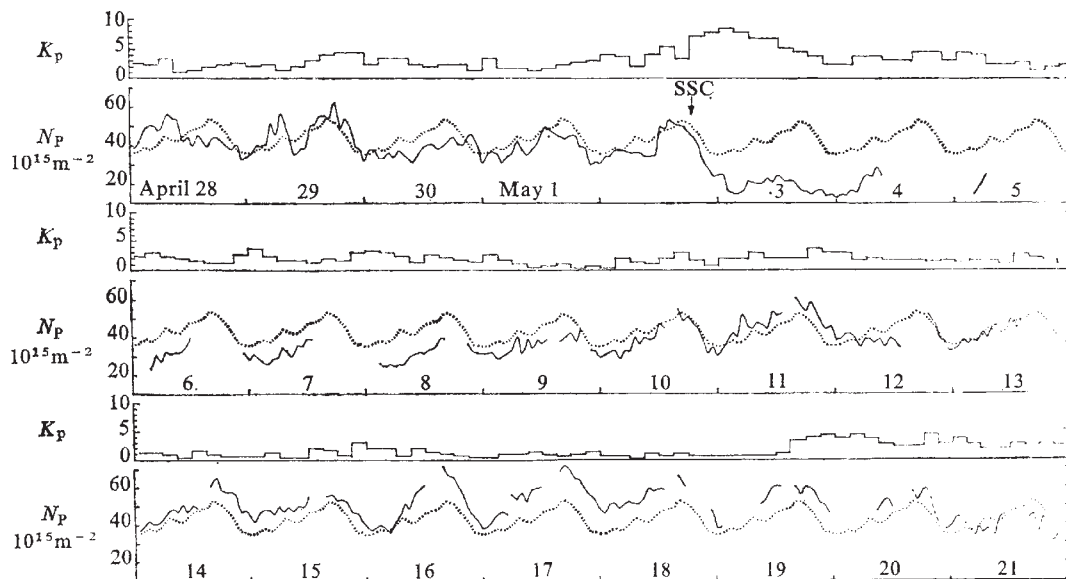


Fig. 1 Protonospheric content variations from 28 April 1976 to 21 May 1976. The dotted curve displays the mean diurnal variation for May 1976 recurring each day. Also plotted are the three-hourly values of the planetary geomagnetic index K_p .

and afternoon maximum, is essentially a quiet time phenomenon. When K_p values exceeding 3 are found over a period of several days the diurnal pattern of N_p becomes irregular with noiselike quasi-random fluctuations which tend to mask the diurnal component of the variation.

Enhanced geomagnetic activity accompanying a sudden commencement is associated with an increase in the daytime protonospheric content. This result is consistent with that reported by Poletti-Liuzzi *et al.*⁷. It is well known that ionospheric electron content can show a significant positive effect during daytime following sudden commencement. This increase is associated with the raising of the ionisation under the influence of the magnetospheric electric field penetrating to the mid-latitude ionosphere and also neutral winds, possibly reversed in direction and blowing equatorwards in response to enhanced temperatures in the auroral zone. It is thus to be expected that the increase in ionospheric plasma will be reflected in enhanced protonospheric content. An additional factor which may cause some enhancement in slant N_p is the expansion of the plasmasphere in the afternoon sector in response to increased magnetospheric convection.

The rapid depletion in N_p as the line of sight moves into the nightside is consistent with the corresponding drop in ionospheric electron content and peak density reported by many workers. The main cause of this latter decrease is almost certainly linked to changes in thermospheric circulation reflected in an increased N_2/O ratio⁸, but the enhanced magnetospheric electric field probably plays a part in depressing the night-time ionisation and thus reducing the ionospheric electron content and hence protonospheric content. The additional factor which contributes to the lowering of the night-time N_p during the main phase of the storm is the contraction of the plasmapause radius caused by the convection electric field. For example, Park⁴ found that the plasmapause moved in to $L \approx 2.4$ several hours after local midnight during a storm in which K_p reached 7.

The slow refilling of the protonosphere after the storm depletion took ~ 14 d to reach saturation during the quiet conditions in May. The results of the whistler studies reported by Park⁴ indicate time constants to reach equilibrium ranging from ~ 1 d at $L=2.5$ to 8 d at $L=4$. The storm of 2 May 1976 with K_p maximising at 8+ was more intense than that discussed by Park so that the protonospheric depletion may have been more complete in our observations where the lowest N_p value ever found occurred, however, in terms of season and solar minimum epoch the data are comparable. Park's observations were interrupted by substorm activity after 8 d so that he was unable to study possible saturation beyond $L=4$. However, in

the present observations the period of quiet extends to ~ 17 d allowing complete equilibrium along the slant path to be reached after ~ 14 d when the plasmapause radius had extended to its furthest possible quiet time position. Murphy *et al.*⁵ calculated a post-storm saturation time for the H^+ tube content which was much greater than the experimental estimate of Park for $L=3$ at solar minimum—they reported a refilling time greater than 8 d at equinox.

After ~ 7 –8 d the N_p magnitudes and their diurnal variation become comparable with the monthly average values suggesting that the median protonospheric content measured by the ATS-6 experiment represents the integration over flux tubes up to $L \sim 4$.

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Possible climatic and biological impact of nearby supernovae

OZONE plays a very important part in atmospheric radiative transfer. The absorption of the solar ultraviolet radiation by ozone is the dominant heating mechanism in the stratosphere. At thermal infrared wavelengths the main ozone contribution comes from the $9.6 \mu\text{m}$ band. Clark *et al.*¹ were unclear, however, whether the supernovae removal of ozone

from the atmosphere of the Earth would heat or cool the surface of the planet. There is evidence, reported here, which enables a more precise statement to be made of the effect upon the global Earth. The reduction in the concentration of ozone will cool the stratosphere, troposphere and surface layers of the Earth.

An estimate of the ozone effect upon the global temperature structure may be made through radiative equilibrium models in which the convective adjustment to the lapse rate is used to approximate the dynamical effects (see, for example, Manabe and Wetherald³). The atmospheric model is one dimensional and is allowed to reach an equilibrium structure with a time marching procedure. For the assumed atmospheric composition and initial temperature structure, the solar and thermal heating/cooling rates are calculated at each level and the net heating/cooling used to determine the temperature at each altitude at time $t + \Delta t$. If the computed temperature lapse rate exceeds the maximum assigned value of -6.5 K km^{-1} , then a convective adjustment occurs with a vertical energy flux sufficient to yield that pre-assigned maximum lapse rate. The time marching procedure is continued until equilibrium is reached at each level in the atmosphere, which is typically 300 d with a time step of 1 d. The final atmospheric structure is independent of the initial temperature profile.

Table 1 gives examples of predicted changes due to the reduction in ozone amount. The small difference in the exact magnitude of the change results from minor differences in the radiative schemes and cloud formulations used by the individual authors. In physical terms a reduction in ozone leads to a decrease in stratospheric solar absorption. The cooling of this region will reduce the downward longwave flux in the $9.6 \mu\text{m}$ band and therefore cool both the troposphere and surface. But the changes in the tropospheric temperatures will be much smaller than the stratospheric effects where ozone plays a dominant part (Table 2).

Table 1 Surface temperature changes due to reduced ozone

Ozone reduction (%)	Temperature change (K)	Ref.
25	-0.34	3
50	-0.3	5
	-0.4	4
100 (No ozone)	-0.5	5

The possible biological consequences of the reduction in atmospheric ozone through these supernovae events will be catastrophic for certain species, as DNA, which carries the genetic information of the cell, is easily modified by exposure to ultraviolet radiation. While living cells have a significant enzymatic capacity for repairing ultraviolet damage to their DNA, living things are in a delicate balance between the continual photochemical destruction of cellular components by solar radiation and their biological repair. If this balance is upset by exposure to increased amounts of ultraviolet radiation which would result from the supernovae events postulated by Clark *et al.*¹, the organism will be injured and may die.

The climatic implications of these supernovae events are also likely to be equally catastrophic. It is generally accepted that a reduction in global temperature of 1K is necessary to produce an ice age (see, for example, ref. 7), which would, of course, hide the larger changes that occur on a local scale. The calculations produced here may not suggest an immediate ice age would follow the complete destruction of atmospheric ozone; but these calculations have been performed with a simple model which does not include the feedbacks that may result from the dynamical

Table 2 Atmospheric temperature changes due to reduced ozone

Pressure (mbar)	Temperature change (K)		
	30% decrease ⁴	50% decrease ⁵	No ozone ⁵
22	-7	-11	-69
230	-0.4	-0.5	-0.6
1,000	-0.2	-0.3	-0.5

coupling between the stratosphere and troposphere. Some insight into these feedback mechanisms has been given by Bates⁸ who suggests the large changes in stratospheric temperature, such as those indicated in Table 2 due to the reduction in ozone amount, would greatly affect the stratospheric meteorology and in particular the static stability of this region. Furthermore, Bates⁸ shows that the ultra-long wave geopotential field in the troposphere is very sensitive to these stratospheric changes indicating a coupling between these atmospheric layers, and a further possible mechanism by which these stratospheric ozone changes would affect the terrestrial climate. There would seem to be little doubt the encounters with nearby supernovae, causing a major reduction in the atmospheric ozone content, would initiate an ice age as Clark *et al.*¹ postulated. This is a further example of an astronomical influence on the terrestrial environment.

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Surface topography as a nonstationary random process

TOPOGRAPHY is often considered as a narrow bandwidth of features covering the form or shape of the surface. After detailed study of many measurements we consider that as well as the possibility of a dominant range of features there is always an underlying random structure where undulations in surface height continue over as broad a bandwidth as the surface size will allow. We consider this a result of many physical effects each confined to a specific waveband but no band being dominant. We invoke the central limit theorem and show through Gaussian statistics that the variance of the height distribution of such a structure is linearly related to the length of sample involved. In another form, the power spectral density, this relationship is shown to agree well with measurements of structures taken over many scales of size, and from throughout the physical universe.

Van Deusen¹ noted that the power spectral densities of a variety of natural and artificial surfaces fall off as the square of the angular frequency. It has since been observed particularly in the longer wavelength irregularities²⁻⁴ that many engineering surfaces exhibit similar spectra. Similarities in the structure of surfaces of such disparate scales of size suggest the existence of a common natural law underlying the phenomena. The specific form of the spectra further suggests that a sample of finite length taken from such a surface will never, however long, completely represent its properties. Real surfaces cannot

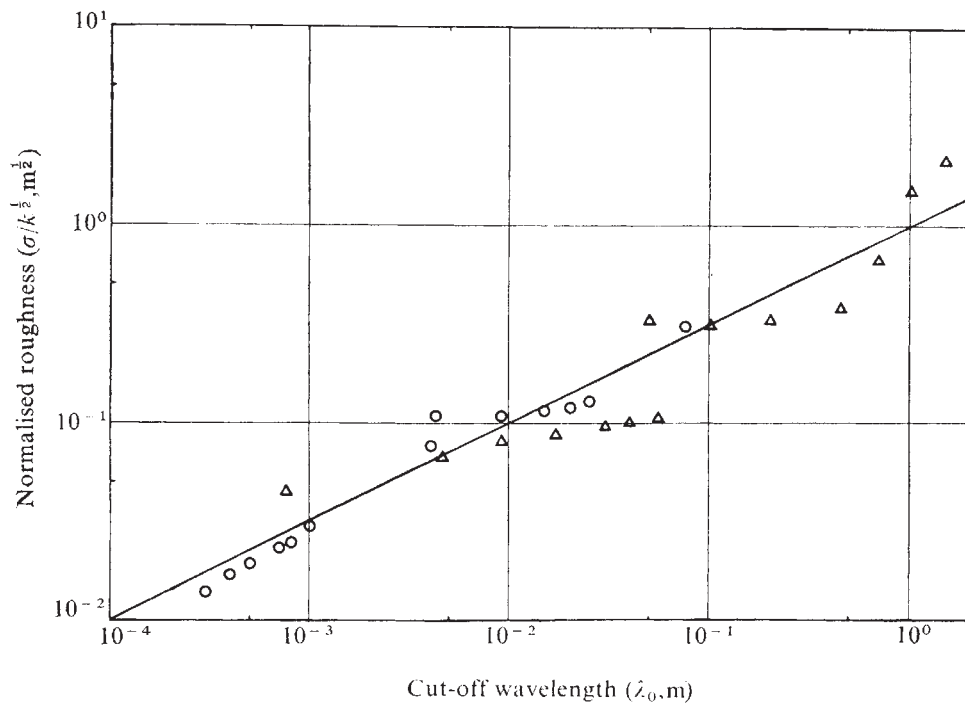


Fig. 1 The experimental points show the increase in normalised r.m.s. roughness ($\sigma/k^{1/2}$, $m^{1/2}$) with cut-off wavelength (λ_0 , m) observed from two different topographies. The solid line represents the theory given by equation (1). $\circ$, Grit blasted surface, $k(m) = 1.7 \times 10^{-8}$ (ref. 7). $\triangle$, Planed surface, $k(m) = 4.4 \times 10^{-12}$ (ref. 2).

therefore be treated as stationary random processes, as has previously been supposed^{5,6}.

A further property of such a spectrum is that its energy increases rapidly with increasing wavelength. The area under the spectrum, which is the variance of the height distribution or, in engineer's terminology, the square of the r.m.s. roughness, is thus most heavily influenced by the longest wavelengths in the sample. This in turn suggests that the roughness must be related in some way to the length of sample or to the measured bandwidth, a hypothesis supported by experimental evidence^{2,7,8} (Fig. 1).

To form such a surface structure we postulate a process resulting from many random causes without preference for any one wavelength or waveband. From the central limit theorem we would expect the amplitude distribution of a sample from such a process to be Gaussian. This is true even if the result of each individual effect forming the process is non-Gaussian. Furthermore, because the process cannot be confined to any one bandwidth, the variance of the Gaussian height distribution will be a function of the length of sample considered, or, equivalently, the bandwidth of features represented.

With a surface of a given finite area, if the heights are measured sufficiently closely together then an arbitrarily large sample can be obtained. Its extreme values, however, would not be very large and will therefore be a function not of the sample size, as for a discrete normal variate, but of the area over which the sample was taken. This is because in the physical world we do not expect things to vary by vast amounts in short periods or distances: a consequence, broadly speaking, of the energy necessary to create large changes in values. Large changes in surface height on Gaussian surfaces are possible but they tend to occur over large distances. This argument suggests that in physical situations a Gaussian stationary process is in fact a contradiction in terms.

Suppose the standard deviation σ of the height distribution is a function of the length of sample L , that is, $\sigma = f(L)$. To investigate the effects of increasing sample length on r.m.s. roughness we will consider consecutive lengths L_1 and L_2 . Wiener⁹, considering the analogous problem of the wandering of a particle in non-overlapping intervals of time, showed that if the two distributions governing the extent of wandering were independent and Gaussian, then the combined distribution is also Gaussian. Thus giving the standard result of the addition theorem that the variances add to form the variance

of the new distribution. The interesting aspect of this result is that in our case, where the standard deviation is a function of sample length then because

$$\sigma^2 = \sigma_1^2 + \sigma_2^2$$

we can write,

$$f^2(L_1 + L_2) = f^2(L_1) + f^2(L_2)$$

and the only function $f(L)$ which holds for the above equation is a linear one,

$$\sigma^2 \propto L$$

The relationship depends on the independence argued between samples, thus with machined surfaces it is restricted to a certain range of features. In general this range is most evident over the spatially longer features of the topography, the smaller features being dominated by the machining marks; however, many natural surfaces have no such restrictions. In such cases the process involved corresponds to the non-stationary Wiener-Levy process¹⁰, of which the best-known example is Brownian motion. Such a process is continuous but nowhere differentiable, confirming the view⁵ that slopes, curvatures and densities of maxima and minima are not, as was formerly widely believed, intrinsic properties of a surface.

This relationship can be expressed in a spectral form, as the effective high-pass cutoff wavelength λ_0 of a sample must be proportional to its length, that is,

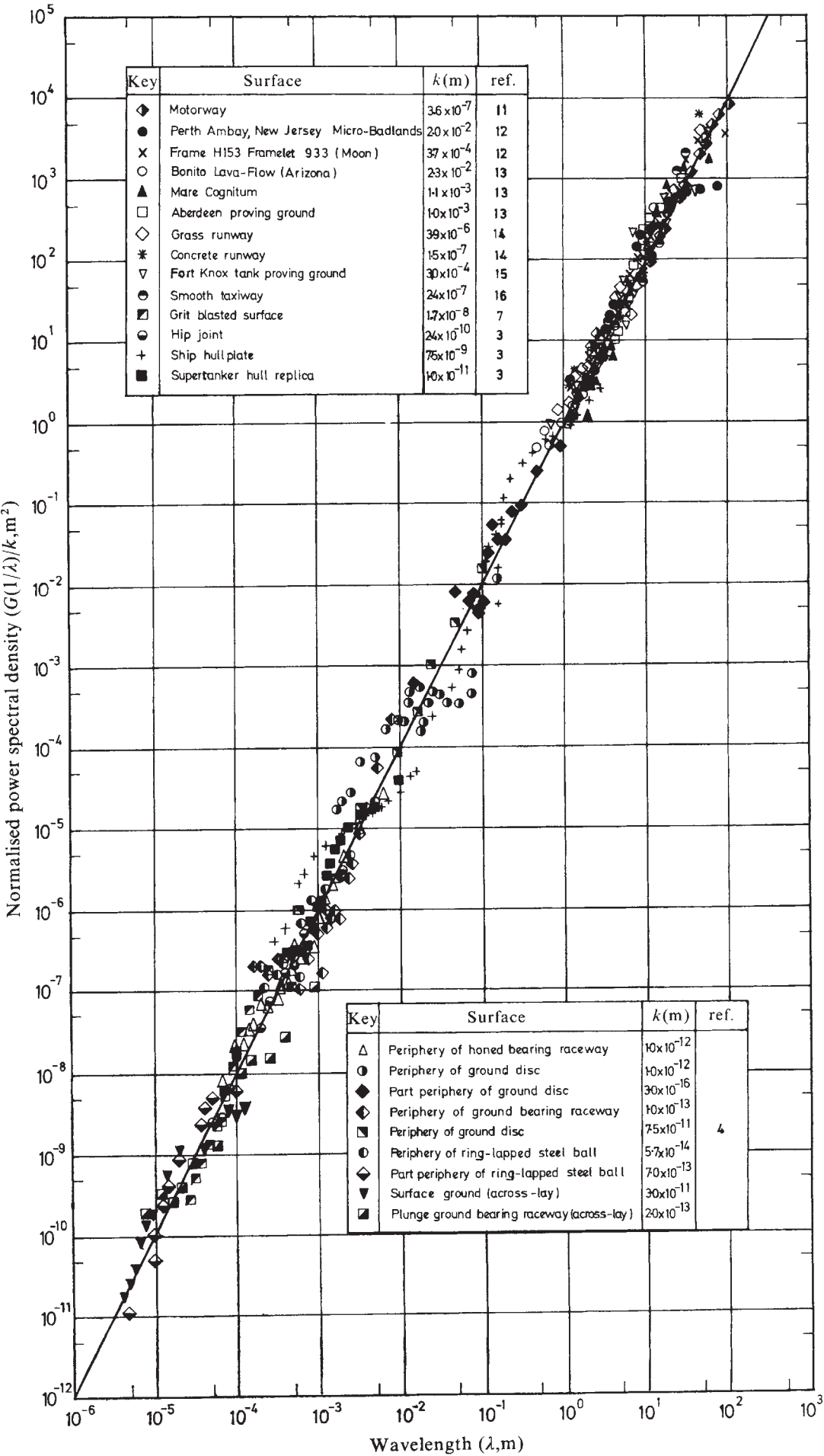
$$\sigma^2 = k\lambda_0 = 2\pi k/\omega_0 \quad (1)$$

where ω_0 is the corresponding angular frequency and k the constant of proportionality, a result confirmed by experiment (Fig. 1). But the area under the power spectral density curve $G(\omega)$ is also the variance of the height distribution:

$$\sigma^2 = \int_{\omega_0}^{\infty} G(\omega) d\omega$$

Combining the above two equations, and assuming

Fig. 2 Variation of normalised power spectral density ($G(1/\lambda/k, m^2)$) with wavelength (λ, m). The graph shows that many different surface topographies existing in the physical universe have a similar form of power spectrum. Note that the spectra available cover almost eight decades of surface wavelength and throughout this range the r.m.s. power increases, to a good approximation, as the square of the wavelength (solid line, equation (2)).



$$\lim_{\omega \rightarrow \infty} G(\omega) = 0$$

which is expected with physical data, then

$$G(\omega) = 2\pi k/\omega^2 = (k/2\pi)\lambda^2 \quad \text{or } G(1/\lambda) = k\lambda^2 \quad (2)$$

in agreement with experiment (Figure 2.).

We call k the 'topothesy' of the surface (τοποθεσία, a description of a place or region¹⁷). Its value uniquely defines the statistical geometry of the random components of an isotropic surface for any given range of wavelengths. The topothesy has units of length. It seems able to characterise successfully and completely, examples of surface structures of a wide range of sizes (Fig. 2) drawn from throughout the physical universe.

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Isotope separation by 'Taylor diffusion'

THE axial dispersion of injected diffusible matter in a fluid flowing in steady laminar regime through a tube has been described by Taylor. On this principle, a simple technical device enabling isotopic separation can be constructed. The separation factor for light stable isotopes is higher than that reached by standard methods; solar energy can be used as an energy source for its functioning.

Taylor^{1,2} and later Aris³ have shown that when a soluble substance is introduced into a fluid flowing through a tube in laminar regime, it spreads out under the combined action of molecular diffusion and the variation of velocity over cross-section. The axial dispersion can be characterised by a virtual coefficient of diffusion (K) which is given by

$$K = (a^2 \bar{u}^2 / 48D) + D \quad (1)$$

if the following condition holds

$$(4L/a) \gg (\bar{u}a/D) \quad (2)$$

D is the coefficient of molecular diffusion of the soluble substance assumed to be independent of its concentration; a is the radius of

the tube, L the distance between the point of introduction and measurement and $\bar{u}$ the mean axial velocity of convection.

If the origin of the coordinates ($z = 0$) is chosen at the point where the substance is injected instantaneously, its axial distribution can be described by the Gaussian curve

$$C(z, t) = \frac{1}{2}(\pi K t)^{-1/2} \exp[-(z - \bar{u}t)^2 / 4Kt] \quad (3)$$

which is the solution of the diffusion equation

$$\frac{\partial C}{\partial t} = K \frac{\partial^2 C}{\partial z^2} - \bar{u} \frac{\partial C}{\partial z} \quad (4)$$

with $C(z, t = 0) = \delta(z)$ as initial condition.

Taylor diffusion has been considered as one of the mechanisms of gas mixing in the human lung. Our previous work on gas transport in the bronchial ducts⁴ has led to a straightforward application of Taylor diffusion to separate isotopes⁵.

Each element of separation (Fig. 1) is essentially formed by a simple tube of length L and circular cross-section of radius a , through which a carrier fluid flows in steady laminar regime. At one end of the tube ($z = 0$), pulses of isotopic mixture are injected periodically with a frequency f . The concentration of a single pulse is given by equations (1) and (3), where D is the molecular diffusion coefficient of each isotopic component in the carrier fluid. Concentration curves must crossover and these intersection points define alternate zones in the tube which are enriched in one or other of the isotopes (bottom of Fig. 1). Heavy isotopes spread more than light ones.

If a valve system is used at point $z = L$, enabling the alternate communication between the principal duct and each of the secondary ducts, and if the valve switch times correspond to crossover concentration points, then each secondary tube will be enriched in one particular isotope.

The separation factor α is given by the classical formula⁶

$$\alpha = \frac{n_{1S}n_{2I}}{n_{1I}n_{2S}} \quad (5)$$

In our case n_{1S} and n_{2I} are the number of molecules of isotope 1 and 2 in the upper (S) and lower (I) secondary ducts respectively (Fig. 1). (We assume $n_{1S} > n_{1I}$.)

In order to produce a fast axial dispersion, we chose parametrical values such that

$$\frac{a^2 \bar{u}^2}{48D} \gg D \quad (6)$$

which, together with condition (2) is consistent with Reynolds numbers ensuring laminar flow. If we call K_1 and K_2 the dispersion coefficients given by equation (1) for isotope 1 and 2 we have

$$\frac{K_2}{K_1} = \frac{D_1}{D_2} \quad (7)$$

Where D_1 and D_2 are the molecular diffusion coefficients of isotopes 1 and 2 in the carrier fluid.

Let m_1 , m_2 and m_0 be the molecular weights of isotope 1, 2 and

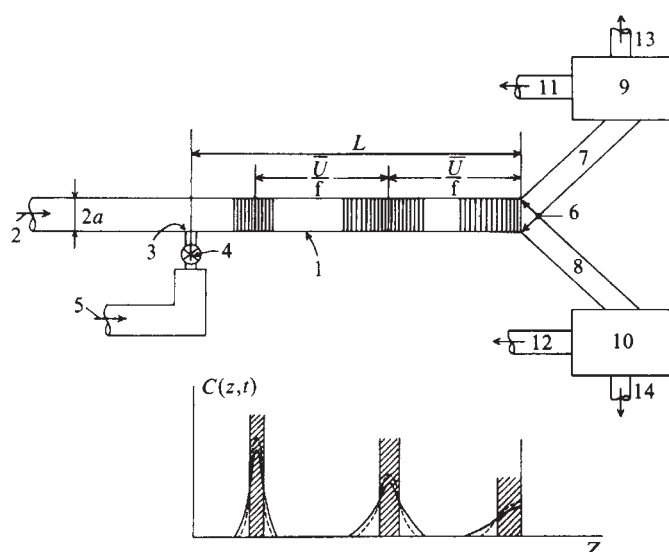


Fig. 1 Isotope separation element: 1, main duct; 2, inflow of carrier fluid; 3, isotopic mixture injection point; 4, injection valve; 5, inflow of isotopic mixture; 6, outflow valve; 7, 8, outflow or secondary ducts; 9, 10, separators of isotopic mixture and carrier fluid; 11, 12, outflow of carrier gas; 13, 14, outflow of isotopic mixture. The concentration of each isotope after $t = L/\bar{u}$ after first injection and expressed as a fraction of the injected concentration. Shaded zones correspond to zones enriched in light isotope.

the carrier fluid respectively. From the Chapman–Enskog classical equation, one can show that

$$K_2/K_1 = (m_2/m_1)^{1/2}[(m_0 + m_1)/(m_0 + m_2)]^{1/2} \quad (8)$$

if $m_2 > m_1$. If $m_1 \approx m_2$, we can write

$$K_2/K_1 = 1 + \varepsilon; \quad \varepsilon \ll 1 \quad (9)$$

When inequality 2 holds, equation (3) can be approximated by a Gaussian distribution and the separation factor is then given by

$$\alpha = 1 + \varepsilon \{ (2\pi e)^{1/2} \operatorname{erf}(1/\sqrt{2}) [1 - \operatorname{erf}(1/\sqrt{2})] \}^{-1} \quad (10)$$

or

$$\alpha = 1 + 1.12\varepsilon \quad (11)$$

Among the different methods of isotopic separation, mass diffusion developed by Hertz^{7,8} also makes use of an auxiliary gas. Later, Maier⁹ used elements consisting of concentric columns separated by a barrier and a feed gas was injected into the innermost column. The faster molecular diffusion of the lighter isotope in the feed gas enables separation.

Both in mass diffusion and in our method, almost all the energy used in the process is related to the separation of the isotopic mixture and the auxiliary fluid. Indeed, in our method, the energy consumption associated with the pipe flow viscous losses is very small. The minimum work required to separate m_1 moles of isotopic mixture and m_2 moles of auxiliary fluid is given by

$$W = RT \left(m_1 \ln \frac{m_1}{m_1 + m_2} + m_2 \ln \frac{m_2}{m_1 + m_2} \right) \quad (12)$$

Where T is the absolute temperature and R the gas constant⁶. However, the proposed method presents three important advantages over mass diffusion: firstly, the simplicity of the element of separation, which is basically constituted by a simple tube; secondly, fluids in their liquid phase can be used, which leads to much smaller plants, and finally, the possibility of having a higher

separation factor. Indeed, in mass diffusion plants, it is recommended that an auxiliary gas and isotopic mixture of close molecular weights be used to avoid slow molecular diffusion⁶. In the present method, because of the inverse relationship between axial dispersion and molecular diffusion (equation (1)), a heavy carrier gas produces a fast dispersion which results also in a higher separation factor. It can then approach the limiting value of $1 + 1.12(m_2/m_1)^{1/2}$.

We have represented infinitesimally small isotopic injections by Dirac functions, in order to get analytical expressions for the separation factor. But, if the mean fractional concentration of the injected mixture in the principal tube is equal to 0.1, numerical integration shows that the separation factor should be multiplied by 0.965.

In Table 1 we present separation factors for isotopes C^{12} – C^{13} given by Benedict and Pigford⁶ for different methods of isotopic separation as well as for the present one. These values are theoretical and can be much smaller in practice. The experimental factor for gaseous diffusion, for example, is about half the theoretical one. Our experimental evidence is sufficiently conclusive for us to believe that in the present method, the experimental separation factor will be much closer to the theoretical one. Indeed, our experiments have shown¹⁰ that the concentration curves, which are not disturbed by the movement of valves, fit equation (3) very well.

Table 1 Comparison of α for separation of C^{12} and C^{13} using different methods

Separation method	Working substances	α
Chemical exchange	HCN–CN [−]	1.013
Distillation	CO	1.010
Gaseous diffusion	CH ₄	1.030
Mass diffusion	CH ₄ –H ₂ O	1.016
Thermal diffusion	CH ₄	1.0026
Gas centrifuge	CH ₄	1.009
Present method	CH ₄ –C ₄ F ₁₀	1.031
(with the mean isotope concentration equal to 10%).		

The separation of stable light isotopes constitutes probably the most interesting short-term application of our method. The energy cost of separation constitutes only a few per cent of the present cost of stable isotopes. The possible application for enriching uranium in isotope 235 is related to the availability of very heavy molecules. Using $N(C_4F_9)_3$ as carrier fluid, this would lead to a separation factor of 1.0031. The construction of an experimental device for separating uranium isotopes is now under study.

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First proof of structure of a C_{28} -pentacyclic triterpane in petroleum

DURING the past decade, C_{27} , C_{28} , C_{30} , and C_{31} modified pentacyclic triterpane hydrocarbons, mainly hopanes, have been discovered in fossil fuels¹⁻⁴. Besides their fundamental importance as diagenetic products from the conversion of living organisms to hydrocarbon deposits in nature, the interpretation of their structure in terms of precursor-product relationships promises to be useful for problems of petroleum exploration^{5,6}. The reported presence of 'degraded triterpanes containing 27 and 29 but not 28 carbon atoms'² has mechanistically been rationalised² in analogy to the observed absence of C_{17} -isoprenoid hydrocarbons in fossil fuels because of the unlikelihood of cleavage of two carbon-carbon bonds located at the same carbon atom (C-22) of the hopane side chain. We determine here the structure of a C_{28} member of the hopane family, 17 α (H), 18 α (H), 21 β (H)-28,30-bisnorhopane (Fig. 1) and its enormous natural abundance (26 p.p.m. of rock) in the Monterey shale, offshore Santa Barbara, California.

After isolation of saturates⁷ from the shale extract, a concentrate containing 50% of C_{28} -triterpane was isolated by preparative gas chromatography⁷. Further purification on acidic alumina 1 (ref. 8) revealed the presence of at least two components in the ratio of 3 : 1 and led to the isolation of $C_{28}H_{48}$ -triterpane-I (Fig. 1), the predominating component, in crystalline form (melting point 150–152 °C), by slow evaporation of a concentrated hexane solution.

Proof of structure of $C_{28}H_{48}$ -triterpane-I as being a bisnorhopane with methyl groups missing at C-18 and C-22 and 17 α (H), 21 β (H) stereochemistry, being 17 α (H)-28-noradientane, was obtained as follows: (1) mass spectrometry: 70 eV, m/e (relative intensity) 384 (95, M^+), 369 (12, $M-CH_3$), 355 (10, $M-C_2H_5$), 191 (100, rings A+B), 177 (20), 163 (30, rings D+E), 149 (15). The observed ratio of intensities at m/e 191 to that at 163 is consistent with 17 α (H), 21 β (H) stereochemistry³. The m/e 355 fragment signals an intact ethyl side chain. The remaining methyl deficiency must, therefore, be one of the angular methyl groups on the triterpane nucleus. The high

abundance of m/e 191 indicates the A and B portion of the hopane ring system intact. The facile cleavage of C-8/C-14 bond to produce the two major fragments at m/e 191 and 163 indicates the presence of 8 β - and 14 α -methyl groups. Thus, the methyl group must have been lost from C-18. The mass spectral evidence by itself, however, does not permit clear distinction from an alternate structure, namely, 28,30-bisnorlupane. Elimination of the latter and confirmation of the structure of $C_{28}H_{48}$ -triterpane-I (Fig. 1) was achieved by X-ray crystallography.

(2) X-ray crystallography: the compound is orthorhombic, space group $P2_12_12_1$, with $Z = 4$. The cell parameters are: $a = 11.26$ Å, $b = 7.36$ Å, $c = 28.38$ Å. 3,090 Reflections were measured on an automatic diffractometer of which 2,242 were considered to be observed. The structure was solved by direct methods, and the data were refined to $R = 0.068$ by block-diagonal least-squares refinement of structure factors. The mean bond distance over 32 values is 1.536 (21) Å; and all distances are within the 2σ limit except the C-8/C-14 distance, which is 1.600 Å, analogous to 1.622 Å in 17 α (H)-adientane¹⁰, explaining the observed facile cleavage of this bond by mass spectrometry.

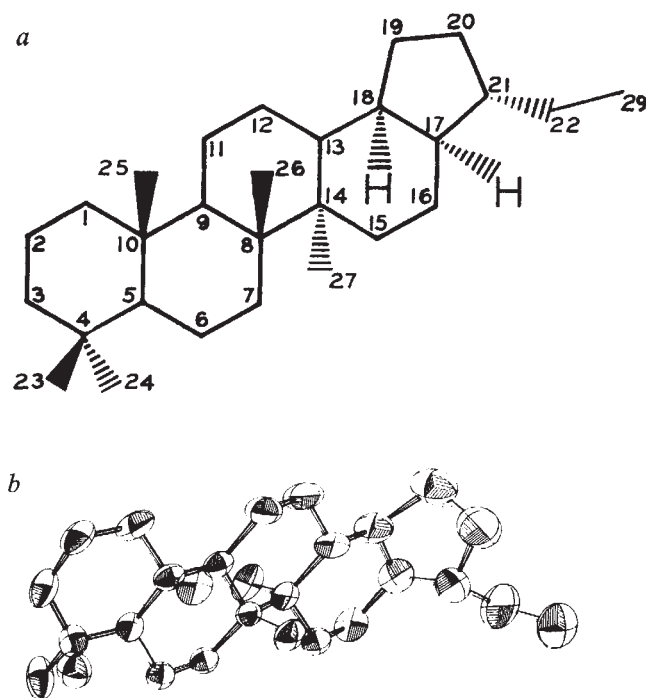
(3) NMR: 360 MHz, ($CDCl_3$) δ 0.793 (3s, 4 β -CH₃), 0.834 (3s, 4 α -CH₃), 0.843 (3s, 10 β -CH₃), 0.886 (3t, $\delta = 7$ Hz, 22-CH₃), 0.899 (3s, 14 α -CH₃), 0.937 (3s, 8 β -CH₃); the chemical shifts of the 4 β , 4 α , and 10 β -methyl group protons are equivalent to those shifts for 17 α (H)-hopane [($CDCl_3$) δ 0.80 (4 β), 0.83 (4 α), 0.85 (10 β)] (ref. 11), and the 8 β -methyl protons are only slightly shifted from those of 17 α (H)-hopane [δ 0.96 (8 β)] (ref. 11) confirming the natural carbon skeleton for the ring A-B portion of $C_{28}H_{48}$ -triterpane-I. The relatively large upfield shift for the 14 α -methyl protons from 1.00 p.p.m. in 17 α (H)-hopane¹¹ to 0.899 p.p.m. in $C_{28}H_{48}$ -triterpane-I is consistent with a relief of steric interaction by removal of the 18 α -methyl group, whose signal is absent in $C_{28}H_{48}$ -triterpane-I [0.91 p.p.m. in 17 α (H)-hopane]¹¹.

The particular C_{28} -triterpane reported here seems to be either absent, or present only in traces, in most fossil fuel samples¹⁻⁴. However, it is present in many California crudes⁶ and occurs with abundance in the nearby Monterey shale and presumably also in large abundance in a crude form from the Northern Volga Ural (Siva) Region (USSR)¹². This points toward some unique diagenetic pathway. Present speculation centres on adipatol¹³, a fern triterpenoid of the 30-norhopane type possessing a hemiacetal linkage between C-22 and C-28. Hydrolysis would yield a CH_2OH -group at C-18 whose oxidation to the corresponding carboxylic acid and subsequent decarboxylation, all accepted geochemical processes¹⁴, would yield the identified hydrocarbon (Fig. 1).

An alternate precursor-product relationship can be visualised starting with adiantone (C_{29}). The latter was postulated¹⁵ to be the precursor for certain partially aromatised hopanes in fossil fuels of proven structure possessing the ethyl side chain in ring E, analogous to $C_{28}H_{48}$ -triterpane-I. The progressive aromatisation required is assumed to begin in ring D. Thus, introduction of the first double bond by reduction of adiantone, dehydration, and shift of the double bond into ring D into the most stable 13(18) position¹⁶ under strongly acidic conditions must result in elimination of the methyl group at C-18. Hydrogenation at this point, parallel or in lieu of aromatisation due to the particular geochemical environment, would yield our $C_{28}H_{48}$ -triterpane-I. Similarly 21-hydroxyadiantone¹⁷, a fern constituent, could give rise to our C_{28} -triterpane through formation of a conjugated diene, accompanied by loss of the methyl group at C-18 and subsequent hydrogenation.

Finally, to explain the unusual abundance of this bisnorhopane in selected cases (Monterey, Siva), there is the remote possibility of a molecular sieve effect in nature, that is enrichment by molecular size due to pore size of the particular inorganic matrix present. Current investigations deal with the structure of the second $C_{28}H_{48}$ -triterpane isomer present in minor quantity.

Fig. 1 Structure of $C_{28}H_{48}$ -triterpane-I. a, 17 α (H), 18 α (H), 21 β (H)-28,30-bisnorhopane; b, stereoscopic view drawn by ORTEP⁸, vibration ellipsoids scaled to 50% probability.



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Strontium isotope evidence for petrogenesis of Mexican andesites

THE Mexican volcanic belt (MVB) consists of several inactive and active volcanoes¹ (Fig. 1). The active volcanoes include Colima^{1,2} and Ceboruco³ near the Pacific coast, Popocatepetl and Iztaccihuatl in central Mexico, and San Martin⁴ close to the Caribbean coast. The MVB rests on continental crust approximately 40 km thick^{5,6}, and is associated with subduction of the Cocos plate below central Mexico^{3,7}. The link between plate subduction and the disposition of the MVB is not simple since the latter makes an angle of about 20° with the Middle America Trench³. This complexity might be related to the structure of the continental crust³, or to a combination of subduction and extensional processes⁴. The most prominent products of volcanism of the MVB are the large composite volcanoes built up from successive eruptions of intermediate lavas and pyroclastic rocks^{3,8}. The lavas of the active volcanoes shown in Fig. 1 are dominantly calc-alkaline andesite and dacite^{3,8–10}. In contrast, the easternmost active volcano, San Martin, is built from lavas of a picritic basalt-basanitoid-alkali basalt-hawaiite association^{4,11,12}. This volcanic area occurs at a change in direction of the MVB and might therefore be linked with extension or fracturing associated with the destructive margin setting of the MVB (ref. 4). Here we report new Sr isotope data for lavas from Ceboruco, Colima, and San Martin. These data, and published Sr isotope data for Cainozoic volcanics from central Mexico¹³ constitute evidence for petrogenesis of Mexican volcanic rocks.

The only published Sr isotope data for Mexican lavas are from rocks of Miocene–Recent age from the area

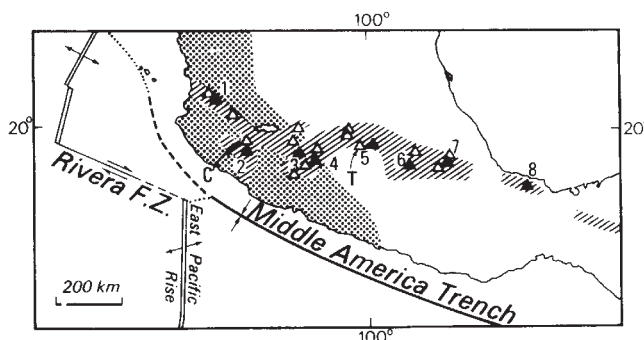


Fig. 1 Volcanoes and plate tectonics of Central Mexico. The two Cainozoic volcanic provinces are shown: the stippled area is the Cordilleran Province and the diagonal shading is the Mexican volcanic belt³. Δ , Inactive composite volcanoes including C, Nevado de Colima and T, Nevado de Toluca. $\blacktriangle$, Recently active volcanoes numbered: 1, Ceboruco; 2, Colima; 3, Paricutin; 4, Jorullo; 5, Xitli; 6, Popocatepetl; 7, Orizaba; 8, San Martin.

around the inactive volcano, Nevado de Toluca^{13,14} (Fig. 1). Whitford and Bloomfield¹³ have obtained Sr isotope ratios in the range 0.7032–0.7045 for 30 samples of lavas from the Toluca area. There is no overall correlation between $^{87}\text{Rb}/^{86}\text{Sr}$ and $^{87}\text{Sr}/^{86}\text{Sr}$, but 10 samples from the immediate vicinity of the volcano show a significant positive correlation indicating an apparent age of 430 ± 97 Myr (ref. 13, p. 211). Whitford and Bloomfield argue that the relatively low $^{87}\text{Sr}/^{86}\text{Sr}$ ratios suggest an ultimate origin in the mantle (ref. 13, p. 212).

We have determined Sr isotope data for 16 Pliocene–Recent lavas from Ceboruco, the Colima area and San Martin. Sr isotope ratios were determined on a VG-Micromass 30 mass spectrometer using chemical and mass-spectrometric techniques described elsewhere¹⁵. Measurements of the Eimer and Amend standard SrCO_3 carried out during this work yield an overall mean of 0.70814 ± 1 . Rb/Sr isotope ratios were determined by a precise X-ray fluorescence technique¹⁶. The Sr isotope ratios and related analytical data for the Mexican lavas are presented in Table 1. Because the analysed samples are all geologically very young (< 1 Myr), the $^{87}\text{Sr}/^{86}\text{Sr}$ ratios presented in Table 1 are taken as initial ratios. These data are shown as a plot of $^{87}\text{Rb}/^{86}\text{Sr}$ against $^{87}\text{Sr}/^{86}\text{Sr}$ in Fig. 2.

Figure 2 shows that the Sr isotope compositions of samples from the Colima area and from San Martin show slight but significant internal variation within the range

Fig. 2 Plot of $^{87}\text{Sr}/^{86}\text{Sr}$ against $^{87}\text{Rb}/^{86}\text{Sr}$ for volcanic rocks from Mexico. $\bullet$, Miocene–Recent lavas from the Nevado de Toluca area¹³; $\circ$, from Colima and Nevado de Colima; $\square$, from Ceboruco; Δ , from San Martin (see Table 1).

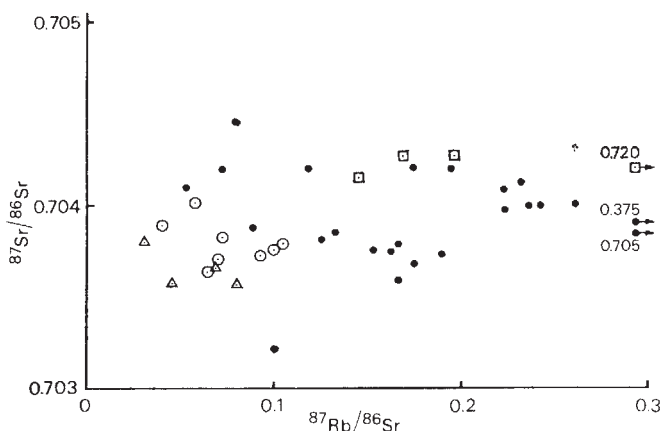


Table 1 Analyses of Mexican lavas

Specimen no.	Rock type	Rb* (p.p.m.)	Sr* (p.p.m.)	⁸⁷ Rb/ ⁸⁶ Sr†	⁸⁷ Sr/ ⁸⁶ Sr‡
Nevado De Colima					
C18	Andesite	19	785	0.072	0.70384 ± 6
C20	Andesite	19	871	0.064	0.70363 ± 6
C27	Andesite	21	648	0.093	0.70373 ± 7
Colima					
C39	Basaltic andesite	24	1,750	0.041	0.70388 ± 5
C40	Basaltic andesite	38	1,932	0.058	0.70402 ± 5
C36	Andesite	20	564	0.104	0.70378 ± 7
C37	Andesite	20	565	0.101	0.70376 ± 7
C109	Andesite	13	557	0.069	0.70369 ± 7
Ceboruco ³					
M43	Andesite	35	527	0.194	0.70427 ± 5
M45	Andesite	35	586	0.171	0.70427 ± 6
M46	Andesite	31	628	0.145	0.70414 ± 5
M42	Dacite	61	246	0.720	0.70420 ± 7
San Martin ⁴					
M71	Picritic basalt	14	1,312	0.029	0.70380 ± 6
M76	Basanitoid	15	663	0.067	0.70366 ± 8
M63	Alkali basalt	19	679	0.081	0.70358 ± 6
M74	Alkali basalt	10	660	0.044	0.70357 ± 6

* Determined by X-ray fluorescence. Mass absorption coefficients estimated from Compton scattering, concentration data about ± 5%.

† Average precision ± 1% (for XRF-techniques, see ref. 16).

‡ Within-run precision quoted as 2 × s.e.m.

0.7036–0.7040 but lie entirely within the range of data for the Toluca area reported by Whitford and Bloomfield (0.7032–0.7045, ref. 13). In contrast, the Ceboruco samples show virtually no significant variation and have ⁸⁷Sr/⁸⁶Sr ratios higher than any data from Colima or San Martin with an average of 0.7042. None of the new sets of data for individual volcanoes show significant correlation of ⁸⁷Sr/⁸⁶Sr with Rb⁸⁷/Sr⁸⁶ (see ref. 17). These data enable us to comment as follows on the petrogenesis of Mexican volcanics.

Values of ⁸⁷Sr/⁸⁶Sr for all analyses of andesitic volcanics fall into the range reported for intermediate lavas from Central America^{18,19}, and for lavas from volcanic arcs where sialic crust is absent^{20–22}. The low Sr isotope ratios for these andesites, commonly in the range 0.7035–0.7040, suggest that sialic contribution to their origin is insignificant.

The mean Sr isotope ratio of 0.7042 of the Ceboruco lavas is significantly higher than that for most of the other samples plotted in Fig. 2, although slightly lower than most continental andesites from South America^{23,24}. These higher ratios could be accounted for by slight contamination of Ceboruco lavas with crustal Sr (compare ref. 23). Ceboruco is also anomalous in showing a high rate of increase in K₂O relative to SiO₂ in comparison with other volcanics from the MVB³. In view of the internal consistency of the Ceboruco Sr isotope ratios, and the similarity of absolute Sr concentrations in Ceboruco lavas to other Mexican andesites (Table 1), it is possible that the higher Sr isotope ratios reflect the Sr isotope composition of the mantle below the volcano. The Sr isotope data therefore suggest a small degree of mantle Sr isotope heterogeneity below the western part of the MVB.

The Sr isotope ratios of the basanites and alkali basalts from San Martin fall into the general range reported for alkali basalts from elsewhere^{25–27}. We suggest that the similarity of Sr isotope values for San Martin basalts with most Mexican andesites indicates a common mantle source.

The Sr isotope data are furthermore consistent with experimental evidence that undersaturated basalt and over-saturated andesite might both be contrasted products of partial melting of mantle peridotite under different conditions of pressure, temperature and activity of water in the coexisting volatile phase²⁸.

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Rapakivi granite, anorthosite and charnockitic plutonism

THE close association of massive anorthosite and charnockitic granitic rocks is well documented¹. Rare earth element (REE) investigations²⁻⁴ have indicated that the massive charnockite (mangerite) associated with anorthosite is not comagmatic with it but represents a distinct magma fraction. Bridgwater *et al.*⁵ describe a charnockitic mantle around a plutonic suite of norites, monzonites and rapakivi granites from the Kap Farvel district of south Greenland which they consider to represent a parallel situation to the anorthosite-mangerite association. They believe contact metamorphic processes accompanied by anatexis produced the charnockitic phases. The charnockites are, therefore, dependent on the associated igneous suites but are not comagmatic with them. Duchesne *et al.*⁷ have investigated the REE content of monzonitic rocks considered consanguineous with the anorthosites of the South Rogaland district of southern Norway. The absence of any Eu anomaly in the monzonitic rocks, as compared with the marked positive Eu anomaly found for the anorthosites, places severe constraints on any postulated common parent magma for this rock association. A magma produced by partial fusion of upper mantle Kaersutite has been suggested⁷. A link between anorthosite and rapakivi granite⁶ has been proposed, rapakivi granite being a high level crystallisation related to and contemporaneous with anorthosite emplacement at greater depth. From their study of the Pikes Peak Batholith, Colorado, Barker *et al.*⁸ proposed a composite model for the origin of the rock spectrum gabbro-anorthosite-syenite-granite (including rapakivi granite). Convecting, mantle-derived, alkaline olivine basalt magma is believed to produce a quartz-syenitic magma from K₂O-poor lower crustal rocks which in turn reacts with the granodioritic to granitic rocks of higher crustal levels to produce biotite and biotite-hornblende granites. Anorthosite is a precipitate phase from intermediate liquids. Our results from an REE investigation of plutonic charnockitic rocks and associated granites from south-west Sweden suggest that rapakivi granite may be associated with the charnockites satellitic to anorthosite rather than with anorthosite itself.

The southern parts of the south-west Swedish gneiss belt have much in common with the south Norwegian anorthosite province although no massive anorthosite has been located in the Swedish area. An intrusive, compound, plutonic association, the Charnockite-Granite Association (C.G.A.), which includes both charnockitic and non-charnockitic components, outcrops in the Varberg area of south-west Sweden⁹. This complex was initiated, mobilised and emplaced during a metamorphism which produced *in situ* charnockitisation within the country rock sequences; meta-arkosic gneisses and a more varied layered sequence with a significant basic metavolcanic com-

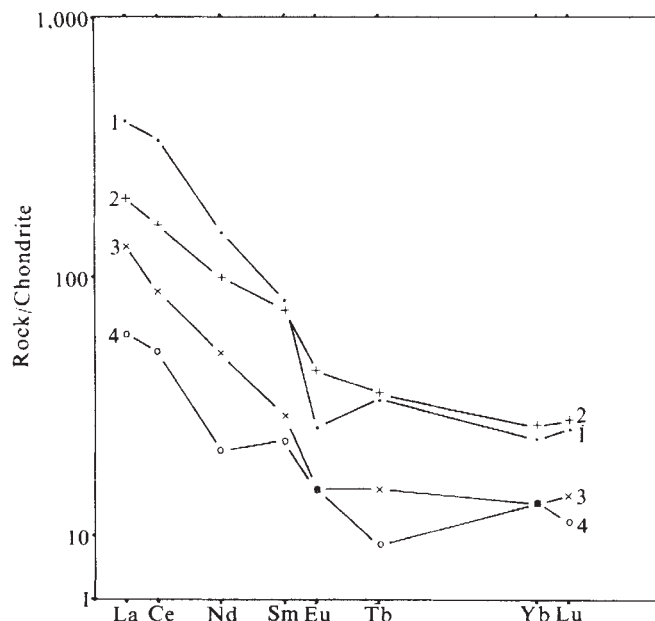


Fig. 1 Chondrite-normalised REE distribution curves. 1. Torpa granite of the C.G.A. 2. Charnockitic members of the C.G.A. 3. Charnockitised granite gneiss, Varberg Formation. 4. Charnockitic quartzofeldspathic granulite, Varberg Formation. Data of Table 1 normalised against Leedey L6 chondrite.

ponent. The admixture of charnockitic and non-charnockitic elements in the C.G.A. is interpreted on the basis of an internal differentiation during emplacement, controlled by H₂O aggregation. The ultimate result of the segregation process was the separation of a differentially mobile, relatively 'wet', granite fraction from a comparatively sluggish, 'dry', intermediate residuum. The latter crystallised as massive charnockite without having travelled far from the site of original anatectic melt generation. By virtue of its differing physicochemical properties, however, the granite differentiates was able to rise further and could separate from its cogenetic charnockite associates. In the Varberg occurrence, however, the link with the charnockites was never completely severed. The advancing head of the granite (the Torpa granite) passed from the granulite sequence, thought to represent reworked basement, to an overlying sequence of layered metasedimentary cover rocks where it spread laterally to crystallise as a series of subconformable sheets⁹. This effectively halted further separation.

The rare earths La, Ce, Nd, Sm, Eu, Tb, Yb and Lu have been determined by instrumental neutron activation analysis for the charnockitic and non-charnockitic elements of the C.G.A. and quartzofeldspathic components of the country rocks (Table 1). The chondrite-normalised averaged distribution curves are presented in Fig. 1. If the C.G.A. is an anatectic derivative from rocks similar to its country rocks, as is believed, a significant accumulation of rare earth elements is involved. All rock groupings show strong differentiation, generally with fairly marked inflexion. The C.G.A. charnockitic rock pattern is similar to that obtained for the Quebec mangerite by Philpotts *et al.* but differs from that found for the massive mangerite of the Lofoten-Vesterdaalen province by Green *et al.*⁴ in that there is no sign of the development of a positive Eu anomaly. It is, however, similar to the pattern for the Moskensøy gneisses marginal to the Lofoten anorthosite⁴. The change from charnockite to non-charnockite within the C.G.A. is marked by concentration of the light rare earth elements La, Ce and Nd, increase in total REE and increase in the differentiation index La/Yb from 12 to 27. The non-charnockitic Torpa granite is further distinguished by the development of a negative Eu anomaly.

In Fig. 2 the chondrite-normalised REE distribution plot for the Torpa granite is compared with some average granite

Table 1 REE concentrations in charnockitic and non-charnockitic quartzofeldspathic rocks from the Varberg area (in p.p.m.)

	La	Ce	Nd	Sm	Eu	Tb	Yb	Lu
1	152	334	106	17.0	3.7	2.0	5.7	0.9
2	77.2	155	70.3	18.4	3.0	1.9	6.5	1.0
3	49.7	85.6	36.3	6.8	1.3	0.9	3.3	0.5
4	22.7	49.5	15.0	5.1	1.3	0.5	3.3	0.4

1. Torpa granite (non-charnockitic) of the C.G.A. (three analyses).
 2. Charnockitic phases of C.G.A. (nine analyses).
 3. Granite-gneiss (meta-arkose) (four analyses).
 4. Quartzofeldspathic granulite (two analyses).

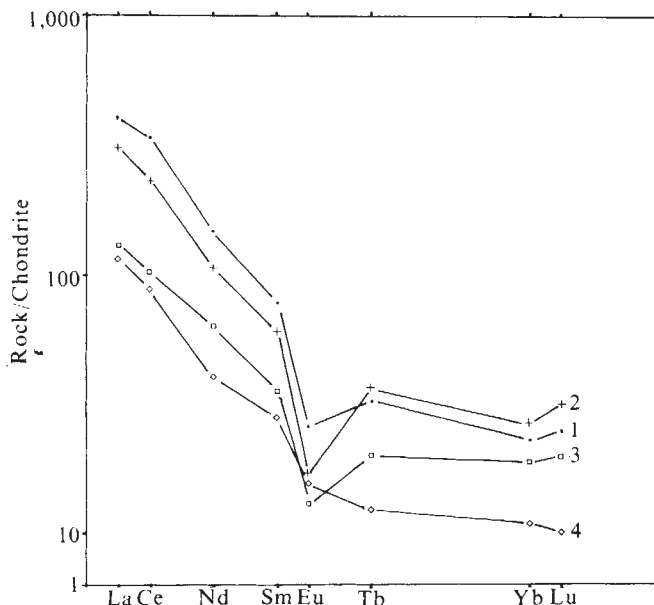


Fig. 2 Comparison of granitic REE chondrite-normalised distribution plots. 1. Torpa granite of C.G.A. 2. Finnish rapakivi granites (after Koljonen *et al.*⁸). 3. 70% SiO₂ granite composite (after Haskin *et al.*⁹). 4. Svecofennian granite (after Koljonen *et al.*⁸).

plots^{10,11}. In both total REE and differentiation style the Torpa granite is strikingly similar to the Finnish rapakivi granites, as reported by Koljonen and Rosenberg¹⁰ and Vorma¹². Identity is marred only by the more pronounced Eu anomaly shown by the Finnish rocks. Both the Swedish and Finnish rocks have REE chemistries which distinguish them from granites of comparable major element composition.

The similar and distinctive REE characteristics of the Finnish rapakivi granites and the Torpa granite of south-west Sweden suggest that they may be similar rocks with a comparable petrogenesis, that is, the Torpa granite is of Finnish rapakivi type. The distinctive mantled megacryst feldspar rapakivi texture occurs, albeit sparingly, within the Torpa granite mass. Although variation in major element chemistry is considerable within rocks recognised as belonging to the rapakivi suite, the SiO₂ range is 64–72%, K₂O is high and MgO always very low⁶. The average SiO₂ for the Torpa granite is 70.7%, K₂O is 5.58% and MgO is 0.52%. The corresponding figures for a rapakivi granite from Satakunta, Finland¹⁰, are: SiO₂ = 68.16%, K₂O = 6.89% and MgO = 0.50%. In contrast, the average values for the charnockites of the C.G.A. are: SiO₂ = 62.73%, K₂O = 4.61% and MgO = 1.51%.

In the Varberg occurrence the relationship of the rapakivi type Torpa granite to the charnockitic plutonism is direct and clearly observable in the field. It is possible that a similar relationship is preserved in the border zones of the large Wiborg rapakivi pluton of south Finland where the red rapakivi granite is reported to grade to dark green rocks akin to mangerite found associated with Precambrian anorthosites⁶. In many cases, however, the granite may have separated entirely from the charnockitic source areas.

On the basis of this study it is proposed that, if a connection exists between rapakivi granite and massive anorthosite, it is indirect. The emplacement of anorthosite in a high-grade crustal environment induces charnockitic recrystallisation and anatectic plutonism in the adjacent country rocks. In some cases, the products of this plutonic activity may differentiate to produce a mobile granitic fraction of rapakivi type which may rise to crystallise at higher crustal levels, sometimes completely dissociated from its source. Rapakivi granite is a product of charnockitic plutonism which in some cases is triggered by anorthosite emplacement.

In the Varberg area no anorthosite is exposed but may exist at unexposed levels. The accession of hot mantle material other

than anorthosite to the deeper crustal levels might be expected to engender charnockite-rapakivi plutonism in a similar manner. There is evidence in the south-west Swedish province of significant ultrabasic and basic magmatic activity contemporaneous with the charnockitisation.

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Apomixis may be widespread among trees of the climax rain forest

THE exceptional species diversity of tropical rain forests is well known; of these the lowland forests of the Malay Peninsula are among the richest¹. This, and the size of the trees, pose obvious difficulties for the maintenance of panmixis, and tempt speculation on the processes by which such diversity has evolved and may now be favoured. Fedorov² suggested that natural selection is low and self-pollination prevalent, favouring random genetic drift. Prevalence of random drift, or apomixis—agamosperry—would contradict Ashton's³ view that, given a stable environment, evolution proceeds through ecotypic differentiation while diversity accrues through ever increasing niche specialisation; for this view to be correct, maintenance of genetic variability within breeding groups would be essential. Solid evidence has been meagre but there is evidence⁴ that genetic polymorphism, and hence heterozygosity, is common in the emergent tree *Shorea leprosula* Miq., and *Xerospermum intermedium* Radlk., an understorey species. The pattern of spatial variation is consistent with the view that these species are outbreeders in which predominantly short-range pollen and fruit dispersal are accompanied by short-range heterogeneity in gene frequencies. However, both are common trees with minimal spatial isolation. We now report the occurrence of apomixis in some other rain forest trees.

Apomixis through adventive polyembryony is well known in cultivated mangoes⁵ whose wild relatives are main canopy trees of the mature phase rain forest, and in cultivated *Citrus*, *Eugenia*, *Garcinia mangostana* Roxb. and *Lansium*^{6–8}, fruit trees which originated from its understorey. We do not know whether such patterns of embryogenesis have arisen after cultivation. Apomixis can also be inferred from triploidy in root-tip squashes derived from several seedlings originating from a single tree of the dipterocarp *Hopea latifolia* Sym. ($2n = 3x = 21$) (misidentified at the time as *H. beccariana*) in Semengoh Forest, Sarawak, West Borneo⁹. A.K. found triploidy also in *Hopea subulata* Sym. ($2n = 3x = 21$), where she also observed that some, or in certain cases all, the embryo sacs abort well before anthesis.

Apomixis can now be demonstrated in the Malayan

emergent dipterocarp *Shorea ovalis* (Korth.) Bl. ssp. *sericea* (Dyer) Ashton, in which, as noted previously, experimental selfings and crossings each lead to similar and relatively high levels of fruit set, and in *S. agami* Wood ex Ashton. *S. leprosula*, with which they grow in mixture, is a diploid with a high level of self-incompatibility in which embryo sac development is of the *Polygonum* type, and embryogenesis is normal. In *S. ovalis* we found that a suite of at least five proembryos develops at the micropylar end from the nucellus; these are therefore almost certainly genetically identical to the parent. Frequently only two seedlings germinate from a single seed and this suggests that only a small proportion of proembryos reach maturity. *S. agami* manifests essentially the same pattern although fewer proembryos seem to be formed. *S. ovalis* examined by us are tetraploid ($2n = 4x = 28$), with meiotic irregularities in the form of univalent and multivalent chromosome associations^{9,10}. The low level of genetic polymorphism reported earlier in this species⁴ may thus be an artefact occasioned by polyploidy.

Table 1 shows that at least one of a syndrome of characters often associated with apomixis, including the occurrence of multiple seedlings in normally single-seeded fruit, high levels of putative self-compatibility, low pollen germinability, irregular meiosis and polyploidy, may occur in rain forest dipterocarps of both canopy and understorey.

Maury¹¹ observed multiple seedlings in, among others, two close relatives of *S. leprosula*: *S. parvifolia* Dyer ($2x = 14$), in which we failed to find any multiple seedlings in

more than 300 seeds examined from another population and *S. argentifolia* Sym. ex Wood ($2x = 14$), in which we found a low percentage. She believed, though on inferential evidence only, that *H. odorata* and possibly also sometimes *S. parvifolia*, *S. ovalis*, *S. argentifolia* and *S. macrophylla* can also exhibit true polyembryony.

The dipterocarp fruit is trilocular and each loculus as a rule has two ovules. Foxworthy¹² noted long ago that several seedlings habitually germinate from fruit of *S. resinosa* Foxw., and we have observed as many as 18; from our counts (several seedlings from one tree) this species is triploid ($2n = 3x = 21$). Other dipterocarps in which multiple seedlings have been recorded include *Dipterocarpus alatus* Roxb., *D. tuberculatus* Roxb. and *Shorea robusta* Gaertn. f., all of which are gregarious species of the Asian seasonal tropics¹², and the semigregarious African savanna dipterocarps *Monotes kerstingii* Gilg, *M. elegans* Gilg and *M. madagascarensis* Humb. (subfamily Monotoideae)¹³. Among 16 Malayan dipterocarp species studied quantitatively by us at least 10 sometimes produce multiple seedlings. It is yet to be confirmed whether in these apomixis is associated with production of multiple seedlings other than in the cases mentioned here; we assume from studies to be published elsewhere that at least in *S. resinosa* and *S. macrophylla*, and it is possible, of course, that adventive embryony occurs in seeds of these and other species in which single embryos develop as well. The percentage of seeds yielding multiple seedlings was estimated in several individuals of *S. agami*, *S. ovalis*, *S. parvifolia* and *S. macro-*

Table 1 Reproductive characteristics of some Malayan Dipterocarpaceae as evidence for apomixis

Species	Chromosome no.	Putative self-compatibility	Pollen germinability	% Seed with multiple seedlings*	Embryological evidence for apomixis	Understorey (U), main canopy (C) or emergent (E)
Evidence not available or negative						
<i>Shorea acuminata</i> Dyer	$2x = 14$			0		E
<i>lepidota</i> Bl.				0	Negative	E
<i>leprosula</i> Miq.	$2x = 14$	Low	High	0	Negative	E
<i>multiflora</i> (Burck) Sym.				0		C
<i>seminis</i> (de Vr.) Soot.				0		E
<i>stenoptera</i> Burck	$2x = 14$			0		U
<i>Dipterocarpus oblongifolius</i> Bl.		High		0	Negative	E
Evidence slight or tentative						
<i>Anisoptera curtisii</i> Dyer ex King				Low†		E
<i>S. argentifolia</i> Sym. ex Wood	$2x = 14$			2 (2)	Tentative‡	E
<i>gratissima</i> Dyer				Low†		E
<i>macrophylla</i> (de Vr.) Ashton	$2x = 14$	Low	High	Low† (2‡)	Tentative‡	C
<i>ovalis</i> (Korth.) Bl. ssp. <i>ovalis</i>				Low‡ (2‡)		E
<i>parvifolia</i> Dyer	$2x = 14$			Variable, low	Tentative‡	E
<i>pauciflora</i> King	$2x = 14$			2 (3)		E
<i>smithiana</i> Sym.	$x = 7$			4‡ (2‡)		E
<i>Hopea mengarawan</i> Miq.				8‡ (2‡)		C
<i>Dipterocarpus baudii</i> Korth.	$2x = 22$			2‡ (2‡)		E
<i>cornutus</i> Dyer				Low†		E
<i>costulatus</i> Soot.				2‡ (2‡)		E
<i>Dryobalanops aromatica</i> Gaertn. f.	$2n = 14$			Low†‡		E
<i>Parashorea densiflora</i> Soot. et Sym.				Low†		E
<i>Vatica pallida</i> Dyer				Low†		U
<i>pauciflora</i> (Korth.) Bl.				Low†		U
Evidence strong but inferential						
<i>S. macrophylla</i> Dyer ssp. <i>macrophylla</i>	$2x = 14$	Low		30–70, variable(9)	Inferential	E
<i>resinosa</i> Foxw.	$2n = 3x = 21$		Nil	98 (18)	Inferential	E
<i>H. latifolia</i> Sym.	$2n = 3x = 21$				Inferential	C
<i>odorata</i> Roxb.	$2n = 3x = 20–22$ §			> 90‡	Inferential‡	E
<i>subulata</i> Sym.	$2n = 3x = 21$		Nil	21 (3)	Inferential	U
Apomixis demonstrated						
<i>S. agami</i> Wood	$2x = 14$		Nil	> 50, variable(4)	Confirmed	E
<i>ovalis</i> (Korth.) Bl. ssp. <i>sericea</i> (Dyer) Ashton	$2n = 4x = 28$ ¶	High		15–47, variable(5)	Confirmed	E

*Maximum number per seed is given in parentheses.

†After Foxworthy¹².

‡After Maury¹².

§Unconfirmed.

¶Irregular meiosis in pollen mother cells.

ptera; in each species some trees yielded a high proportion and others few or none.

Maguire¹⁴ has inferred apomixis in *Clusia* (Clusiaceae) from Guyana. One of us (C.O.H.) has obtained successful fruit set on bagged female inflorescences of the wild dioecious understorey fruit tree *Garcinia parvifolia* Miq. of Western Malesia, in the same family. It is not yet confirmed whether embryos develop from the inner integument as in the cultivated mangosteen (*G. mangostana*)⁷. This species has a high, probably polyploid chromosome number ($2n = 60$).

Though it can hardly be claimed that this preliminary evidence adequately represents a tree flora which in the lowlands of Peninsular Malaysia alone runs into thousands, it does conclusively demonstrate the occurrence of apomixis in both emergent and understorey trees of the mature phase. Further, if the close relationship between the small genus *Lansium* and the large *Aglaia* is accepted, all the cases demonstrated are from the series of closely allied species which occur together in the same habitat and which distinguish Malesian from other tropical forests. Although it is premature to consider these series as themselves evidence of apomictic reproduction comparable to *Hieraceum* or *Rubus*, it is no longer possible to discount the contribution of apomixis to the evolution of some of them and to the high floristic diversity characteristic of lowland tropical rain forest. Western Malesia seems to have had a more uniform climate during the Pleistocene¹⁵ than other continental regions of the humid tropics. If we assume that natural selection in these forests is dominated by biotic factors¹⁶ and that immigration and extinction are continuing, we must conclude that the selective factors are constantly changing, and that few apomictic biotypes will survive for prolonged periods. This suggests that series of allied taxa which include apomicts are evidence for continuing active diversification.

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Regularity versus irregularity in specific songs of closely-related drosophilid flies

THE songs of most insect species are stereotyped and show regular rhythms^{1,2} which can be readily defined and described. In such cases, closely related species produce songs which differ in rhythmic structure³⁻⁵, carrier frequency^{5,6} or combinations of these parameters^{3,7}. We have examined the songs produced during courtship of the sibling drosophilid fly species *Zaprionus tuberculatus*, *Z. sepsoides* and *Z. mascariensis* which are known from morphometric⁸ and chromosomal evidence to be closely related. Drosophilid songs are produced by wing beating and normally consist of a regular series of similar mono- or poly-cyclic sound pulses with carrier frequencies of 150 to 500 Hz and pulse periods of 8 to 250 ms (for review, see ref. 9). Some species produce more than one type of song, and the pulsed song may be interrupted by protracted bursts of constant frequency tone¹⁰.

Flies were lightly etherised on the day of emergence and the two sexes were isolated in groups of 10 to 30 into vials containing fresh standard food: 10-15 d later, one individual of each sex was placed in a 12 × 12 × 20 mm wire gauze cage within 5 mm of the ribbon of a Grampian GR4 microphone (for further information on the acoustics and methods see refs 9, 10). During courtship, flies were watched through a binocular microscope and sounds were monitored through headphones as they were produced. Sound records were made at between 18 and 20 °C using amplifiers onto a Tandberg 3341X tape recorder and subsequently oscillograms were made using a Nihon Kohden PC 2 B moving film camera.

Records were analysed by direct measurement from the oscillograms of pulse period. In addition, the number of pulses making a discrete sequence, separated by at least 1 s from adjacent sequences or by a change of character of 5 pulses duration were measured. Individual sequences were analysed as follows: the mean period and its standard deviation were calculated. From these, variance ratios were obtained and Student's *t* test was applied between the pulse periods in song sequences produced by different individuals of a species and between pulse periods in the songs of two species. In addition, the Kolmogorov-Smirnov test for exponentiality (*D* test)¹³ was applied to pulse periods in individual sequences of the song of all three species.

Subjectively, the songs of both *Z. tuberculatus* and *Z. sepsoides* sounded very regular, with long uninterrupted sequences of regular sound pulses. Analysis showed that each species produced two songs, a short period song which was similar in both species and a long period song of significantly longer period in *Z. sepsoides* than in *Z. tuberculatus* (Table 1). In both species, the variability in pulse period within a sequence of song of either type was similar. The type 1 song of *Z. tuberculatus* is produced in longer sequences than that of *Z. sepsoides*, but sequence duration is highly variable in both species.

By contrast with the songs of the other two species, that of *Z. mascariensis* always sounded erratic. The duration of a sequence was hard to define and the pulse period within a sequence was highly variable (Table 1). Individual long sequences were tested to find whether the pulse period was random (the *D* test does not discriminate well with short sequences). Of 22 sequences thus analysed, produced by seven individuals, 12 did not differ significantly from randomly produced sequences. All of the other 10 sequences, while showing some pulses produced with a fairly regular short period, also contained

Table 1 Comparison of parameters of songs produced by three species of male *Zaprionus* (Drosophilidae) during courtship at 18–20 °C

Species	Mean pulse period $\pm\sigma$ (ms)	Type 1 song No. of sequences measured	Mean sequence duration $\pm\sigma$ (ms)	Mean no. of pulses per sequence	Mean pulse period $\pm\sigma$ (ms)	No. of sequences measured	Type 2 song Mean sequence dura- tion $\pm\sigma$ (ms)	Mean no. of pulses per sequence $\pm\sigma$
<i>Zaprionus tuberculatus</i> (7)	12.37 $\pm$ 1.32 <i>n</i> = 183	7	730 $\pm$ 531 <i>n</i> = 10	68.3 $\pm$ 50.9 <i>n</i> = 10	26.64 $\pm$ 3.48 <i>n</i> = 240	12	897 $\pm$ 694 <i>n</i> = 10	28.7 $\pm$ 20.7 <i>n</i> = 10
<i>Zaprionus sepsoides</i> (8)	11.23 $\pm$ 1.90 <i>n</i> = 356	14	325 $\pm$ 173 <i>n</i> = 15	29.2 $\pm$ 15.83 <i>n</i> = 15	41.95 $\pm$ 5.21 <i>n</i> = 284	13	1372 $\pm$ 630 <i>n</i> = 15	31.9 $\pm$ 12.96 <i>n</i> = 15
<i>Zaprionus mascariensis</i> (7)	—	—	—	—	81.69 $\pm$ 101.14 <i>n</i> = 880	36	2267 $\pm$ 2162 <i>n</i> = 36	24.9 $\pm$ 21.4 <i>n</i> = 36

Even though the song of *Z. mascariensis* differs from both song types in the other two species, it is treated for the purposes of comparison here as if it were type 2 song. Numbers in parentheses after specific names indicate the individual flies recorded.

long series of erratic pulse periods which were never found in the songs of the other two species. These nonrandom song sequences had a shorter average pulse period and less variability of pulse period than the random sequences (Table 2) but were very significantly more variable than either type of song of the other two species. Although it appears from Table 1 that the duration of song sequences is longer and more variable in *Z. mascariensis* than in the other two species, this is complicated by the fact that in the other species, complex sequences of type 1, then type 2, then type 1, 2–1–2 and several longer combinations have been observed.

It has been shown that the songs of various closely related

which will accept ever more random signals and mechanisms of motor patterning which override the normally rhythmic flight¹⁹ and song-producing mechanisms²⁰.

Irregularity versus regularity as a specific character of insect song has apparently not been previously observed and is thus a rare phenomenon. The situation may be explained as follows: if regularity has evolved, the production and recognition templates will tend to co-evolve and to tighten the song regularity; this is commonly observed. If irregularity once evolves through relaxing of the selective pressure it will tend to proceed to the point of randomness when challenged by nonrandomness; this is what we describe here. Once randomness has evolved, how-

Table 2 Characteristics of song sequence of male *Zaprionus mascariensis*

	No. of sequences observed	Mean pulse period and s.d. (ms)	Mean sequence duration and s.d. (ms)	Mean no. of pulses per sequence and s.d.
Random sequences	12	104.8 $\pm$ 107.2 <i>n</i> = 390	4082 $\pm$ 2685 <i>n</i> = 12	33.6 $\pm$ 22.2 <i>n</i> = 12
Nonrandom sequences	10	53.3 $\pm$ 77.8 <i>n</i> = 405	2319 $\pm$ 1210 <i>n</i> = 10	40.7 $\pm$ 15.2 <i>n</i> = 10
Short sequences	14	111.4 $\pm$ 132.4 <i>n</i> = 85	675 $\pm$ 532 <i>n</i> = 14	6.07 $\pm$ 3.12 <i>n</i> = 14

Short sequences have been taken as those composed of 2–12 pulses which cannot readily be tested for randomness. Nonrandom sequences have a probability of less than 0.05 using the Kolmogorov–Smirnov *D* test¹².

Drosophila species differ in pulse period^{4,13}, and that this difference is an important parameter in courtship success of the male fly and in species isolation^{14,15}. Doubtless such differences are important in distinguishing the rhythmic songs of *Z. tuberculatus* from those of *Z. sepsoides* but it seems that the best character of the song of *Z. mascariensis* by which it can be distinguished from that of the other species is its greater variability and irregular structure. Several advantages have been suggested for the variable song repertoire of birds: that they may be important for individual recognition, in sexual selection or in territorial behaviour (for review see ref. 16). Bird songs, however, do not sound like random sequences, nor are the individual phrases usually as simple as the song pulses of drosophilid flies. Irregularity of the type found in the song of *Z. mascariensis* implies that the female fly has a recognition mechanism capable of accepting irregular sequences and of rejecting regular sequences.

The normal insect situation seems to be that the recognition–production template (for a review of this concept, see ref. 17) is selected to accept ever more stereotyped songs which differ greatly from those of related species from which sexual isolation is desirable¹⁸; this is seen in the type 2 songs *Z. tuberculatus* and *Z. sepsoides* which differ at a level of probability of more than 10^{–6}. By contrast the songs of *Z. mascariensis* which seem to be selected for variability versus regularity will require templates

ever, it is in itself a stable situation from which it is hard to evolve and which therefore is likely to become extinct rapidly and to be rare.

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Semicircular canals in squids

THE cephalopods and fishes are rivals as fast-moving predators and they show many parallel adaptations¹. We present here evidence that in squids and cuttlefishes the statocysts possess semicircular canals, though of rather imperfect form. Animals that move quickly need to monitor angular rotation in order to allow appropriate adjustments of the eyes as they turn. To provide this facility certain critical physical requirements must be met and semicircular canals provide this. Their small size ensures that during rotation the pattern of flow is dominated by viscous damping. Consequently the actual volume of fluid flow is small, permitting "accurate transduction of the volume displacement of fluid by means of a water-tight 'swingdoor' cupula having limited angular excursion in the ampulla"². Furthermore, in vertebrates, the low Reynolds number of the system (less than one) ensures that "the velocity of relative flow becomes strictly proportional to the inertial force driving it and hence to the angular acceleration of the head". The statocysts of the fast-moving cephalopods have gone some way to meet these requirements by their shapes, and by the arrangement of the strange anticristae, projections that partly divide the cavity.

The crista of the statocyst is orientated in several planes³ and is sensitive to both angular and linear acceleration⁴. Further investigation now shows that in squids and cuttlefishes the cavity is shaped to direct the flow of endolymph along specific channels, across which the cupulae of the cristae are placed (Fig. 1). We have studied the canals after fixation and staining

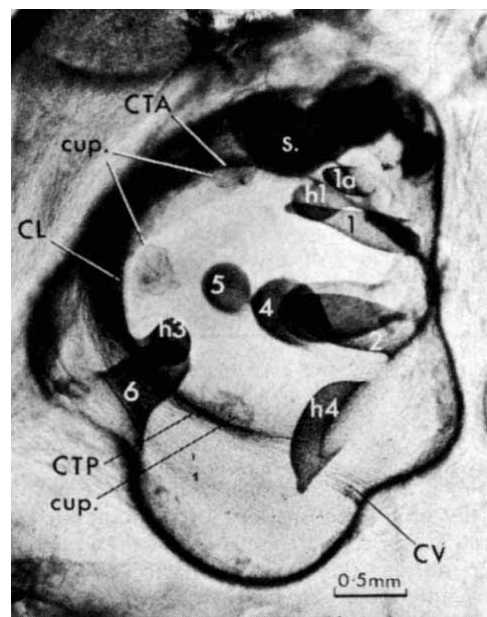


Fig. 2 Left statocyst of *L. vulgaris* stained with haemalum. Seen from above it shows the cupulae on the cristae.

the inside of the statocysts by injection of haemalum or of ink, and by reconstructions from serial sections.

The canals are not complete tubes but are grooves, made by bulges in the outer walls of the sac. The canals are partly enclosed by the projections of cartilage that have been called anticristae⁵. These projections are of two sorts⁵ (Figs 1 and 2). Some are placed at the turns of the crista, where they bend over as hooks, and these have been called hamuli and numbered h1-h5. Elsewhere the anticristae are straight projections (numbered 1-7) with various shapes, arranged in rows so as to divide the cavity into channels. In an adult *Loligo* some of the anticristae become joined together to make complete tunnels, restricting the flow of endolymph (Fig. 3). Some of the anticristae become shaped and grooved along the lines of flow (Fig. 3).

The most fully developed canal, with a nearly complete tunnel, is the horizontal one, related to the vertical section of the crista and thus monitoring angular accelerations to right or left (yawing). These turns about the vertical axis, produced by the animal's own steering, obviously require corresponding adjustments, especially of the eyes. Movements in the rolling plane, monitored by the longitudinal crista, are smaller and not initiated by the animal itself. The corresponding canal does not have any part that forms a complete tunnel. However, this canal is more complete in *Sepia* than in *Loligo* because in the former there is a large hamulus at the first turn of the crista which is absent in *Loligo*. The anterior transverse crista, along the front of the cavity, monitors movements in the pitching plane and is activated by the endolymph in a deep channel above and below it, but again without a covered tunnel.

The function of the posterior transverse crista is more difficult to decide. It may be activated by movement in the pitching plane, as indicated by the arbitrary lines shown in Fig. 4. Possibly the two transverse cristae serve separately to monitor movement in the two directions, head up and head down, a function that is performed in the other planes by the paired statocysts. However, we suggest that it may (also?) serve to monitor linear accelerations, both forwards and backwards⁶. Squids and cuttlefishes have special need for receptors to monitor these movements produced by their jets. No fish has equal powers of movement in both directions. The channel behind this crista extends far backwards and the slope in front of it is gently downwards. This hypothesis is supported by the fact that the crista faces upwards (Fig. 4) so that its cupula

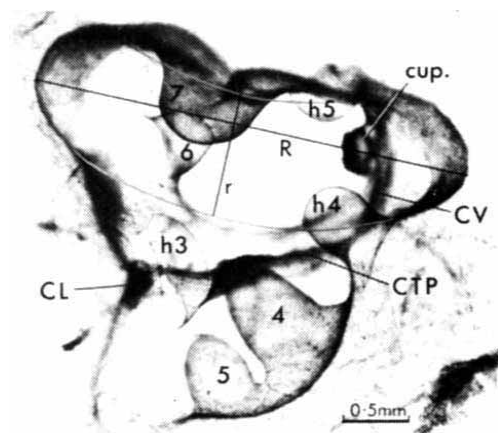


Fig. 1 Horizontal canal of *Loligo vulgaris* of 14 cm mantle length. Left side, seen from behind. The cupula is stained with diluted squid's ink. In all the figures the anticristae and hamuli have been given the numbers used by Dilly, Stephens and Young³. CTA and CTP are the anterior and posterior transverse cristae; CL, longitudinal crista; CV, vertical crista; cup., cupula; n.cr., crista nerve; Kö., Kölliker's canal; s., statolith; h1-5 are the hamuli, and the straight anticristae are numbered 1-7.

stands more nearly vertically than those of the anterior transverse and longitudinal cristae, which are horizontal (Fig. 2).

The details of the statocysts vary with the habits of the animals. The anticristae are all more smoothly rounded in *Sepia* than in *Loligo*. *Sepioteuthis* is a squid which resembles *Sepia* in shape, but, of course, is without a cuttlebone. Its anticristae are also rounded, like those of a cuttlefish; perhaps this form is a result of smooth rotatory movements with the fins.

The anticrista at the front of the statocyst (number 1) is rounded and single in *Sepia* but pointed and double in *Loligo* (Fig. 4, numbers 1 and 1a). In the small, inshore, bottom-living squid *Alloteuthis* this anticrista varies greatly between individuals, sometimes forming a plate, sometimes a whole crowd of little knobs. This region lies in the path of movement of endolymph in several planes (Fig. 4) and anticrista 1 is evidently different from all the others. In *Loligo* it is covered with sensory hairs⁵, and perhaps these are receptors serving to monitor acceleration in several planes. *Alloteuthis* has a long rigid spike or 'tail' and this animal may make movements that impose special requirements on the statocyst and produce the varied knobs at the position of anticrista 1.

In cephalopods that turn slowly, such as octopods, or decapods that live in deep waters, the anticristae are reduced^{7,8}. No doubt this is because sensitivity to slow movements requires a large inertial mass of fluid. Conversely, detection of rapid changes in acceleration would be impaired by a large mass, and the rapidly moving decapods avoid this by developing the anticristae and forming partial tubes.

It is interesting to compare the dimensions of the canals of decapods with those of fishes. The radius of curvature (R) of the horizontal canals of four large *Loligo forbesi* gave a mean value of $R = 1.25$ mm. These animals were all around 37 cm in length and weighed about 1 kg (in air). In fishes of this weight the radius was about 5 mm (ref. 9). The internal radius (r) of the channel in the squids, at its narrowest point, was about 0.4 mm, whereas in the fishes it was 0.2 mm. Interpretation using weight for comparison is obviously difficult because of differences in buoyancy. In the cephalopods the small value of R may compensate for the fact that the canals are incomplete and r is

Fig. 3 Thick section of the right statocyst of a large *Loligo forbesi* (48.5 cm mantle length). Seen from the lateral side, to show the fusion of anticristae 2 and 3 and 6 and 7 to make a nearly complete horizontal canal. Anticrista 7 has become elongated along the line of flow. The cupula is very lightly stained and has been touched up. Stained with haemalum.

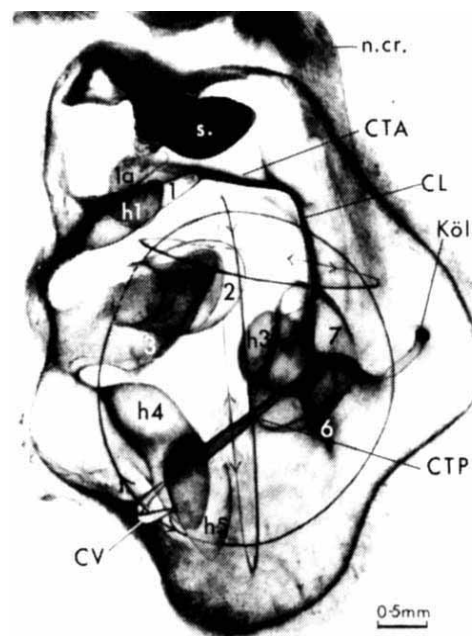
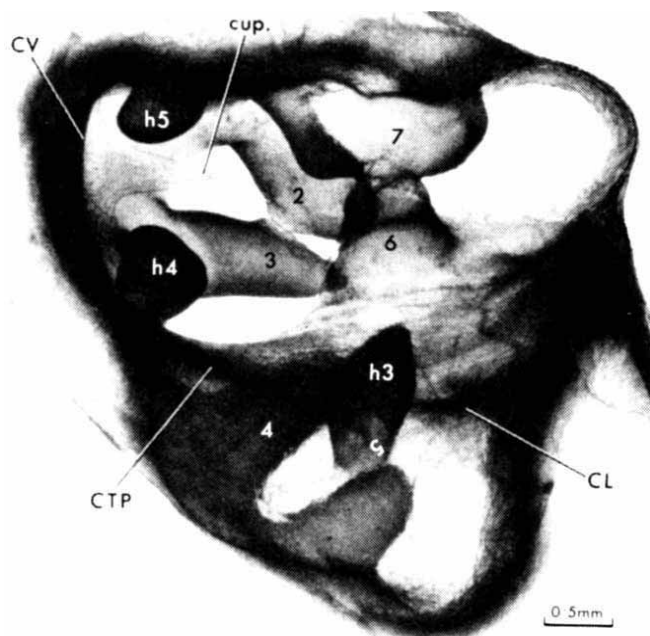


Fig. 4 Right statocyst of a large *L. forbesi* (37 cm mantle length). Seen from above. The presumed directions of flow are shown by arrows. Stained with haemalum. The photograph has been retouched to show the crista more clearly.

large. However, nothing is known of the elastic and other properties of the cupulae, and it is not known whether they allow any flow of fluid past them. Like Budelmann we have seen some appearances suggesting that there may be attachments of the cupulae to the hamuli, perhaps serving to make the channels more nearly 'water-tight'.

It is clear that these devices for canalising flow in the statocyst have the same function as the semicircular canals of vertebrates and the somewhat similar arrangements in Crustacea¹⁰. We must, therefore, add one more to the long list of parallels between the functional organisation of cephalopods and vertebrates¹.

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Development of eye alignment in cats

WHEN the eyelids of newborn kittens open, casual observation of the pupils suggests that the eyes must be divergently misaligned. Pupillary divergence may be quantified by photographing the eyes while the cat is facing a distant light source. The images of this light, formed by reflection at the corneas, coincide approximately with the optical centres of the eyes, and the distance by which the separation of the pupils exceeds the separation of these images is defined as pupillary divergence¹⁻⁵. It declines with increasing age until some time during the second month². Although it is reasonable to assume that the visual axes are also divergent before this age, this assumption leads to a paradox because surgical misalignment of the eyes of young kittens is known to disrupt binocular connections in striate cortex⁶. Moreover, development of normal orientation selectivity in kitten cortex may require early coordinated binocular vision⁷. We have examined this problem using a combination of techniques with which we are able to determine the alignment of the visual axes in the alert cat. Our results show that while the pupillary axes are quite divergent during the first few weeks after birth, the visual axes are not divergent. It is thus possible that kittens experience synchronised binocular vision very early in life.

The four steps required in the method we use are illustrated in Fig. 1. First, the cat is photographed while alert, as shown in (a). An aperture, positioned in front of a tungsten light source 2.4 metres from the cat, produces a small, well-defined corneal image in each eye. Second, within one day, the cat is prepared for electrophysiological study of single neurones in the visual cortex. The paralysed cat is placed in a stereotaxic

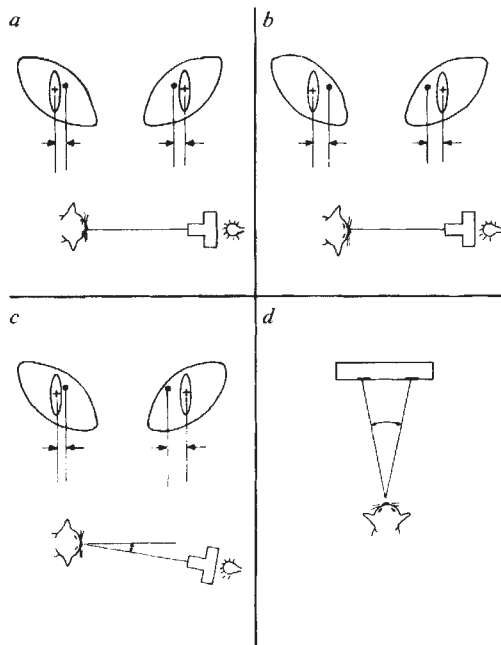


Fig. 1 The steps required to obtain visual axis alignment in the conscious cat. In humans the visual axis or line of sight is defined by a line from the fovea of the retina to the object of fixation passing through the nodal plane of the eye (the nodal plane defines equal magnification between object and image space). In cats we define the visual axis as a line from the centre of the area centralis through the nodal plane to the object of regard. In (a) an alert cat faces a camera and a light source (bottom). The distance by which each pupil centre (+) diverges from the corneal image of the light source (●) is shown (top). The cat is prepared for physiological study and photographs are again taken as shown in (b). To obtain the factor for converting linear into angular divergence, photographs are taken with the camera and light source rotated 10° along an arc (c). Finally, the animal is prepared for single-cell study of striate cortex. The angle between the visual axes is then determined by plotting binocular receptive fields as shown in (d).

assembly so that its position is nearly the same as when it was alert and several photographs are again taken, as shown in (b). Third, in order to find the factor for converting linear divergence of the pupils into angular divergence of the pupillary axes, the camera and the light source are shifted 10° along an arc from the previous position, as depicted in (c), and another series of photographs is taken. The fourth step (d) is to plot the receptive fields for several binocular cells recorded from the striate cortex. The mean distance between the centres of these receptive fields is used to determine the angle between the visual axes.

Animals are prepared for physiological study by standard techniques. Anaesthesia is induced with Fluothane, a vein is cannulated, and anaesthesia is maintained with Brevital (sodium methohexital). A tracheal tube is inserted, scalp bone and dura mater are removed, and tungsten-in-glass electrodes are inserted at the crown of the post-lateral gyrus to penetrate in Area 17 within the representation of the area centralis. The animal is paralysed and maintained with a Flaxedil (gallamine triethiodide)-glucose solution. Peak expired CO₂, electrocardiogram and electroencephalogram data are monitored continuously. Spikes from single neurones are amplified and displayed by standard means. Optimal stimuli are determined for each cell and receptive fields are plotted for individual binocular neurones in the visual cortex.

Negatives are processed, mounted in slide holders, and magnified by projection to enable accurate measurements of pupillary divergence. An exact magnification factor is obtained for each photograph by use of a ruler positioned just above the cat's head in the plane of its corneas.

Using the procedure described in step 1, we have measured the angle between the pupillary axes at successive stages of development for a number of kittens. Figure 2a shows these data for five animals. As found previously², there is marked divergence in the youngest kittens which decreases, but does not entirely disappear, with increasing age.

In order to measure visual axis alignment during this period, we have used the four-step procedure described above. Each of 21 animals was studied once at a particular age. By plotting receptive fields (step 4 above), we are able to measure the angle between the visual axes in the paralysed animal, and since we know how interocular alignment has changed between alert and paralysed states (steps 1-3 above), we are able to infer visual axis alignment in the awake cat. Results of this determination are shown in Fig. 2b. Each open circle represents the angle between the visual axes of an alert cat of a given age. The result is markedly different from that which might be anticipated on the basis of measurements of pupillary divergence. From the earliest age, 2 weeks, all cats are convergent rather than divergent. The magnitudes of convergence are somewhat surprising. This may indicate that alert cats converge excessively under our experimental conditions or that a small systematic error is introduced by our method. In either case, two conclusions may be firmly drawn. First, no trend is evident which suggests that visual axis alignment changes with age. Second, for some positions in object space, cats seem able to receive corresponding binocular input at all ages tested.

If visual axis alignment is relatively constant with age but the angle between the pupillary axes changes substantially, then the angle between the visual and pupillary axes must also vary. This angle is given for cats of different ages in Fig. 2c. Each point represents the angle of pupillary axis divergence if visual axes are assumed to be parallel. These data show a close match between the magnitude and time course of the anatomical changes and the differences in pupillary divergence indicated in Fig. 2a.

Before drawing conclusions from these findings, we must consider a few potential difficulties in our methods and the controls devised to deal with them. First, use of the pupil as a reference for mensuration depends on the assumption that its position remains constant throughout our procedures. Constriction may not be symmetrical however, and pupil centres may shift when the necessary local and systemic paralysing and

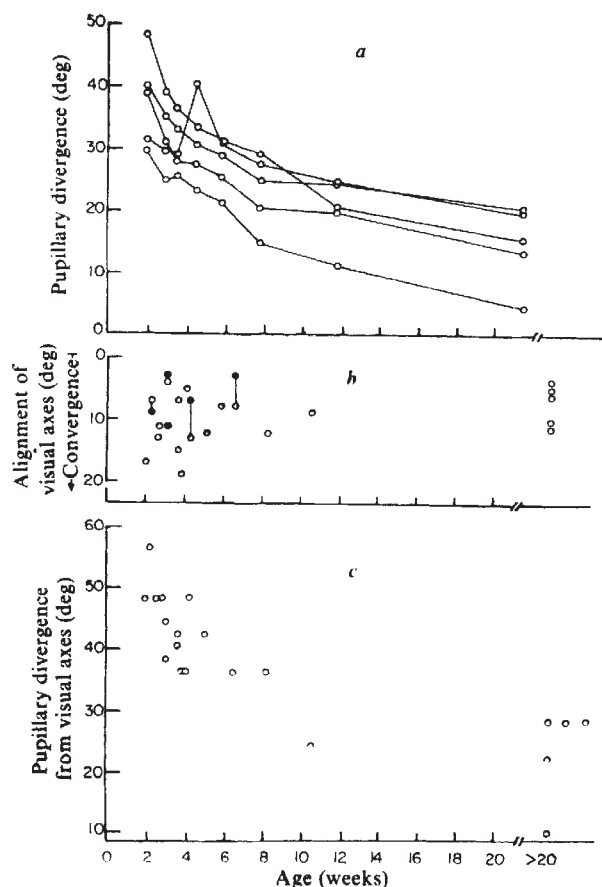


Fig. 2 a, The pupillary axis is defined by a line perpendicular to the cornea that passes through the centre of the eye's entrance pupil (the entrance pupil is the image of the real pupil formed by the cornea). The angle of divergence between the pupillary axes may be obtained from the amount of pupillary divergence measured using photographs. By taking photographs at several angles relative to the cat, we have determined that linear and angular divergences are related by a constant factor (mean for all cats: 15.4 deg mm^{-1}). With the conversion factor and photographs taken before and after immobilisation, it is possible to deduce the angular change in pupillary alignment, and thus in visual axis alignment. Angular divergence of the pupillary axes of the alert kitten decreases with increasing age, as shown for five kittens photographed at regular intervals during development. The distance in mm by which separation of pupil centres exceeds separation of corneal reflexes is converted into degrees through use of the empirically determined conversion factor mentioned above. Each point represents a mean value based on eight photographs. **b,** The angle between the visual axes is operationally determined as follows. The electrode is placed within the Area 17 representation of area centralis. The receptive fields of cells in this region, because they represent homologous retinal points, are separated by the same distance on the plotting screen as those from the centre of the area centralis. Thus the angle between the visual axes is the inverse tangent of the distance between receptive field centres minus inter-pupillary distance divided by the distance between the cat's eyes and the stimulus screen. Visual axis alignment is convergent in cats of all ages. $\circ$, Represent values based on the four-step procedure illustrated in Fig. 1. $\bullet$, Depict values obtained by a method in which corneal tattoos were used similarly to the pupils as eye alignment reference points. In cases where pupils and tattoos were used for one animal, $\bullet$ and $\circ$ are joined by a vertical line. **c,** Angular divergence of pupillary axes relative to visual axes (that is pupillary divergence if the visual axes were parallel) decreases with increasing age. Each point is obtained from a single paralysed animal through combined use of receptive field and photographic measurements. Magnitude and time course of the anatomical change illustrated here match those of the change in pupillary divergence shown in (a) above. Data points in (b) and (c) were obtained from the same animals.

ophthalmic drugs are used. Even small changes could cause considerable errors in determining interocular alignment. To provide an alternate fiducial point to the pupil, very small tattoos made with titanium oxide in saline were placed on the corneas of several cats. Measurements of visual axis alignment based on the use of these tattoos are included in Fig. 2b (filled symbols). When both the tattoo and pupil techniques are used for the same cat, the two points are connected by a vertical line (open to filled symbols). It is clear that the data obtained using tattoos are in agreement with those of the other method. Thus, it seems that no systematic errors result from the use of pupils.

Another possible source of error is concerned with the influence of luminance level. To obtain accurate measurements using pupils, it is necessary to cause constriction by use of high luminance or drugs or both. It is possible that the light levels we use may influence our results. In particular, reflex convergence might occur, and this possibility must be thoroughly explored, as we find that cats are somewhat convergent at all ages. Using tattoos, we were able to take photographs with room illumination only as well as under higher luminance levels (from 50 to $3,000 \text{ cd m}^{-2}$), and we determined that no significant light-induced convergence occurred.

Apart from these considerations, our procedures involve other potential sources of error. Cyclotorsional changes could confound measurements of strictly lateral displacements. Eye drift could occur between the time that photographs of the immobilised animal are taken and the time receptive fields are plotted. Contact lenses, which are inserted before receptive field study, could cause displacement of the eye or prismatic changes of the visual axis. We have ruled out each of these possibilities. Cyclotorsional alignment has been measured under all conditions, and our findings are reported elsewhere⁸. Possible effects due to eye drift and contact lens use have been determined

to be negligible by photographing several times during paralysis for some cats, monitoring eye positions through ophthalmoscopic projection of the optic discs, and by plotting receptive fields with and without contact lenses in place. Sources of variability inherent in our measurements include those related to photographic processing and the plotting of receptive fields. We have analysed each of these factors and find that the overall variability in our procedures is exceedingly small.

We conclude that the visual axes are not divergent in young, alert kittens. This finding is contrary to what may be inferred from pupillary photography alone, and also from receptive field separation in very young paralysed animals⁹. Because the visual axes of young kittens are convergent, it seems likely that fused binocular vision of objects at some distances occurs during early life. Further work is required to confirm this speculation.

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Fatty acids, aldehydes and alcohols as attractants for zoospores of *Phytophthora palmivora*

ZOOSPORES of the important plant pathogenic fungi *Pythium* and *Phytophthora* are attracted to plant roots and to exudates and extracts from roots¹. Attraction (positive chemotaxis) to amino acids^{2,3} which occur in root exudates, and ethanol⁴, which will be produced by roots in waterlogged soils, has been demonstrated, but no substance has been found which by itself is as powerful an attractant as root exudate. The attractiveness of root exudates must therefore be explained by additive or synergistic effects between the known attractants, or by powerful attractants which have hitherto escaped detection, or both. There is evidence for additive and synergistic effects⁵, but the fractionation of root exudates has not so far yielded evidence of additional chemotactic factors. We report here the existence of further attractants for fungal zoospores, and consider the possibility that they may be of significance in chemotaxis to plant roots.

Our experiments were carried out with *Phytophthora palmivora* (Butler) Butler, which attacks a range of tropical crops, especially cocoa (*Theobroma cacao* L.), in which it infects pods, roots and other parts⁶. Chemotaxis of zoospores to cocoa root exudates has been demonstrated⁷, as well as to pod extract⁸ and some amino acids^{2,9}. We used, under licence from the Ministry of Agriculture, Fisheries and Food, the same strain that we used in a study on negative geotaxis of zoospores⁸, and a swim-in test in which the number of zoospores entering a capillary tube containing a suspected attractant are counted and compared with numbers entering a control tube¹. Attraction of *Ph. palmivora* zoospores to some amino acids^{2,9} was confirmed, and in addition they were shown to respond, as do *Phytophthora cinnamomi* zoospores¹, to ethanol. Other alcohols, aldehydes and fatty acids were investigated. Allen and Newhook⁴ had mentioned that *Ph. cinnamomi* showed chemotaxis to 5 mM solutions of methanol, *n*-propanol, *n*-butanol and acetaldehyde, but of these compounds we find only acetaldehyde active with *Ph. palmivora*. Several alcohols, aldehydes and fatty acids, however, were shown to be attractants, some of them having thresholds considerably lower than those for ethanol or any other attractant so far established for fungal zoospores (Table 1). The most potent attractants had 4–6 carbon atoms, iso- compounds tended to be more effective than those with straight chains, and acids and especially aldehydes were usually more powerful than the corresponding alcohols. After entering the capillary tube, zoospores soon settled, encysted and germinated, although some compounds at high concentrations caused lysis instead of encystment. Isovaleraldehyde, for example, resulted in lysis at 10 mM, some lysis but some encystment and germination at 1 mM, and complete germination in the range 0.001–0.1 mM. We have also found that zoospores of *Ph. palmivora* are repelled by H⁺ and other monovalent ions, as are those of *Ph. cinnamomi*¹². Taking into account these repellent effects, experiments on adjusting the pH of attractants have been carried out. These experiments indicate that the fatty acids are attractive in the unionised (R.COOH) but not the ionised state (R.COO⁻). Hence, the new attractants have the common feature of volatility.

Are these new attractants likely to be of significance in nature? In order to ascertain whether such compounds are important in chemotaxis to plant roots, it would be necessary to fractionate root exudates before chemotactic tests in a way that avoids the loss of volatile components. The procedures so far used are unlikely to avoid such losses and hence volatile attractants might have been overlooked.

Table 1 Results of chemotaxis tests on zoospores of *Phytophthora palmivora*

Carbon atoms	Chemical*	Response†	Threshold (mM)‡
Alcohols			
1	Methanol	0	—
2	Ethanol	+	5
3	Propanol	0	—
3	Isopropanol	+	1
4	Butanol	0	—
4	Isobutanol	+	1
4	Tertiary butanol	+	1
5	Amyl alcohol	+	5
5	Isoamyl alcohol	+	0.1
9	Nonanol	0	—
Aldehydes			
2	Acetaldehyde	+	5
3	Propionaldehyde	+	1
4	Butyraldehyde	+	0.1
4	Isobutyraldehyde	+	0.1
5	Valeraldehyde	+	0.1
5	Isovaleraldehyde	+	0.001
6	Caproaldehyde	0	—
7	Heptanal	0	—
8	Octanal	0	—
9	Nonanal	0	—
Organic acids			
2	Acetic	0	—
3	Propionic	0	—
4	Butyric	?	—
4	Isobutyric	?	—
5	Valeric	+	0.5
5	Isovaleric	+	0.5
6	Caproic	+	0.5
6	Isocaproic	+	0.1
8	Octanoic	0	—

Zoospore suspensions ($1-2 \times 10^5$ spores ml⁻¹) were prepared as previously reported⁸ except that the suspending medium into which spores were released was 0.5 mM potassium phosphate buffer (pH 5.8) – CaCl₂ (0.05 mM) – MgCl₂ (0.05 mM). Capillary tubes (1 µl) were filled with the substance under test dissolved in suspending medium and adjusted if necessary to pH 5.8 with 1 M NaOH, or the suspending medium alone (control) by heat sealing one end of the capillary and allowing the liquid to be drawn into the tube as it cooled¹⁰. A test and a control capillary were placed at opposite ends of an assay chamber (20 × 6 × 1 mm deep) which was then filled with a zoospore suspension and covered with a coverslip. After 10 min, spores were immobilised by gently warming the chamber, and the spores inside the two capillaries were counted. Five replicates were carried out with each concentration of a substance tested and the probability of any higher count obtained with the test capillary as compared with the control capillary being due to chance was determined by means of the *t* test for paired samples¹¹.

*Except where indicated as iso- or tertiary, the straight-chain (*n*) compounds were used.

†Attraction by concentration 10 mM, with $P < 0.01$; 0 = no attraction; ? = apparent attraction towards, but not into, capillary.

‡The lowest concentration of an already proved attractant giving attraction with $P < 0.05$.

Fries¹³ states that with gas chromatography pea roots give 'a rich spectrum indicating the presence of numerous volatile compounds' and cites references establishing the production of volatile alcohols, aldehydes and organic acids (including valeric acid) by plant roots, fruits and other parts. Hence the compounds shown in the present study to be chemotactic may well be present in fresh root exudates and have a role in attraction. This may be true of related compounds, such as unsaturated fatty acids, which are known to be produced by roots¹³ but which we have not tested.

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Cell division factors (cytokinins) from irradiated plant tissue

REPORTS on the effects of ionising radiation on plant cells in callus culture are quite limited. Work on a few species^{1–3} has shown that low doses of γ radiation, starting at 0.5 krad, stimulate callus growth and differentiation. Larger doses ranging from a few krad to about 20 krad inhibit growth and differentiation, respectively. High doses (16 krad and above) have been shown to alter culture media components chemically and thereby affect cell growth⁴ and differentiation². We now report that

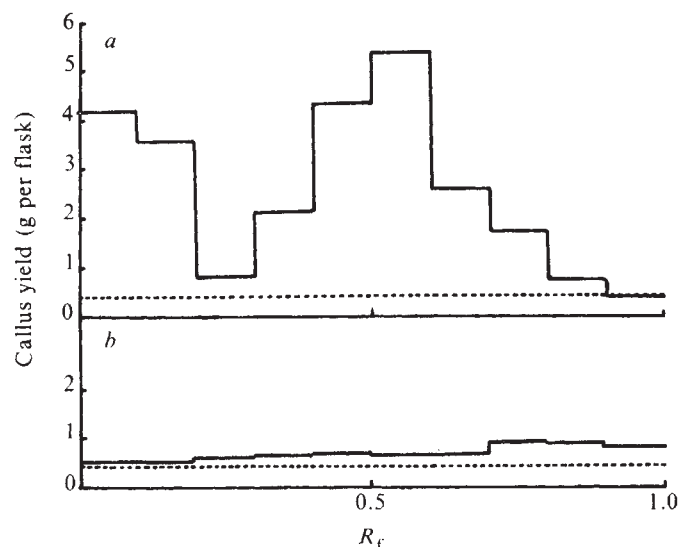


Fig. 1 Tobacco callus cytokinin bioassay of paper chromatographic R_f zones from extracts (140 g equiv. fresh weight) of irradiated (1–2 krad) (a) and nonirradiated (b) *Haworthia* tissue. Tissue (0.4 kg) was blended in a Waring blender with sufficient cold absolute ethanol to obtain a final ethanol concentration of 70% assuming all the tissue to be water. The mixture was shaken in the cold for 8 h and filtered through gauze. The solid residue was resuspended in additional cold 70% ethanol, shaken for 4 h and filtered. Filtrates were combined, adjusted to pH 2.5 with HCl and passed through a Dowex 50W-X8 cation exchange column (2.9 × 15 cm, 50–100 mesh, H⁺ form). The column was washed with 150 ml of distilled water and cytokinins were eluted with 150 ml of 2M NH_4OH followed by 350 ml of 6M NH_4OH . Ammonia was removed from the eluate under an air stream and the remaining aqueous solution was taken to dryness in a vacuum. The dry residue was extracted with 95% ethanol and the ethanolic solution was reduced to a small volume and strip loaded on Whatman 3MM paper. Ascending chromatography was carried out overnight using 2-butanol: concentrated (28% NH_3) NH_4OH (4:1, v/v) and chromatograms were dried, cut transversely into 10 equal R_f strips and eluted with 95% ethanol. Aliquots from each strip were bioassayed for cytokinin activity. Samples for bioassay were dried in a vacuum in 50-ml flasks; 25 ml of nutrient agar medium^{5,9} was added to each, and the flasks were autoclaved (20 min at 18 pounds per inch²). Three pieces of Wisconsin 38 tobacco pith callus (approximately 150 mg per piece) were placed in each flask and the cultures were maintained in diffuse light at $23^\circ \pm 2^\circ \text{C}$. After 5 weeks, tissues were collected and fresh weights were recorded. The broken lines show callus yield on basal bioassay medium; kinetin at $5 \mu\text{g l}^{-1}$ yielded 3.01 g of tobacco callus.

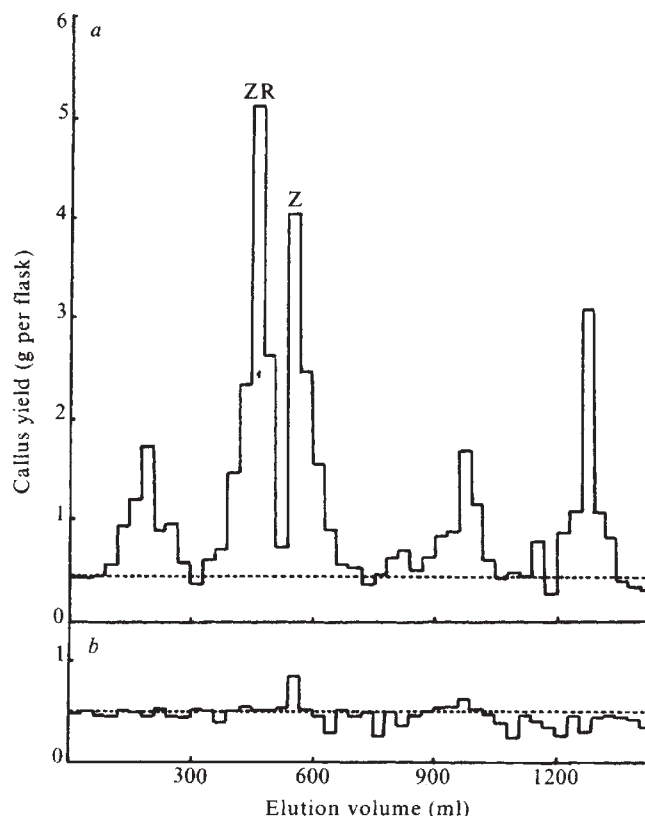


Fig. 2 Tobacco callus cytokinin bioassay of Sephadex LH-20 column fractions (25 g equiv. fresh weight) after chromatography of paper R_f zones 0.4–0.8 (Fig. 1) from irradiated (a) and nonirradiated (b) *Haworthia* tissue. Eluates of paper R_f zones 0.4–0.8 from irradiated tissue were taken to dryness and the residue was dissolved in 1 ml of 35% ethanol and loaded onto a Sephadex LH-20 column (2.5 × 90 cm). The column was eluted with 35% ethanol and forty-eight 30-ml fractions were collected and bioassayed for cytokinin activity according to the procedure described in Fig. 1. Authentic samples of zeatin and zeatin riboside were chromatographed in the same conditions and their elution positions are indicated by Z and ZR, respectively. The broken lines show callus yield on basal bioassay medium; $5 \mu\text{g l}^{-1}$ kinetin yielded 3.42 g of tobacco callus.

gamma irradiation (1–2 krad) of *Haworthia* in callus culture results in the formation of cytokinins⁵—cell division factors—not detected in nonirradiated tissue.

Tissue was excised from surface sterilised inflorescence stem segments of *Haworthia mirabilis* Haw. and cultured on Murashige–Skoog medium⁶ supplemented with synthetic cytokinin (kinetin, 6-furfurylaminopurine, 0.2 mg l^{-1}) and auxin (naphthaleneacetic acid, 0.2 mg l^{-1}). Callus which developed was maintained by subsequent subculture on the same medium containing kinetin and naphthaleneacetic acid (both 1.5 mg l^{-1}). All tissue was kept at $23^\circ \pm 2^\circ \text{C}$ under continuous light.

Callus tissues weighing approximately 350 mg were irradiated using a ^{60}Co γ source. Tissue given 1–2 krad at a dose rate of $0.19 \text{ krad min}^{-1}$ grew continuously, became compact and did not differentiate. It could be subcultured repeatedly on hormone (cytokinin and auxin) and *myo*-inositol-free media and showed no diminution of growth. Nonirradiated callus required hormones or *myo*-inositol for continued growth.

Cytokinins were isolated from irradiated tissue 8–10 weeks after third generation subculture on hormone and *myo*-inositol-free medium by a procedure similar to that described for *Populus × robusta* leaves⁷. Cytokinins were assayed as described in the legend to Fig. 1. Nonirradiated tissue cultured for 8–10 weeks on medium containing kinetin and naphthaleneacetic acid (both 1.5 mg l^{-1}) was analysed for cytokinins in the same way. Figure 1 shows results of cytokinin bioassay of extracts of irradiated and nonirradiated tissue after paper chromatography. There were two zones of activity from irradiated tissue, one at

R_f 0.1–0.2 and a larger response from R_f 0.4 to 0.8. No corresponding response was obtained from nonirradiated tissue. R_f zones 0.4–0.8 from both irradiated and nonirradiated tissue were further purified on a Sephadex LH-20 column using 35% ethanol¹⁰, as described in the legend to Fig. 2.

Five areas of cytokinin activity were obtained from irradiated tissue after chromatography on Sephadex LH-20 (Fig. 2a). The two major peaks centred in fraction 16 and 19 cochromatographed with authentic samples of zeatin riboside (6-(4-hydroxy-3-methyl-2-butenylamino)-9- β -D-ribofuranosylpurine) and zeatin, respectively. Authentic samples of 6-(3-methyl-2-butenylamino) purine and its riboside, both known cytokinins, did not cochromatograph with any peaks of cell division activity from irradiated tissue. Essentially no cytokinin activity was obtained from nonirradiated tissue except a small response in fraction 19 which cochromatographed with zeatin (Fig. 2b). Bulk fractions (200–300 ml each) beyond those shown in Fig. 2 were collected until a total effluent volume of approximately 2,200 ml had passed through the column in the case of the irradiated and of the nonirradiated tissue. Bioassay of these fractions showed no additional cytokinin activity eluting from the column. Chromatography of R_f 0.1 and 0.2 from irradiated tissue on Sephadex LH-20 using the same conditions as described in Fig. 2 yielded a single peak of cytokinin activity centred on fraction 14. This compound eluted before authentic zeatin riboside, our earliest eluting standard cytokinin.

In conclusion, our findings demonstrate that gamma irradiation of plant tissue (*Haworthia*) in callus culture results in the formation of cell division factors or cytokinins. The origin of these compounds and the reason for the diversity of cytokinins in irradiated tissue remain to be clarified. It has been proposed¹¹ that free cytokinins are produced by a pathway that includes synthesis and degradation of cytokinin-containing transfer RNA. The cytokinin content of transfer RNA from irradiated and from nonirradiated tissue is being investigated.

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Chromosomal rearrangement involved in insecticide resistance of *Myzus persicae*

POPULATIONS of the peach-potato aphid *Myzus persicae* (Sulzer) heterozygous for a particular translocation between autosomes 1 and 3 were first discovered in glasshouses in Great Britain¹. A similar or identical translocation was subsequently found to occur commonly in field populations of *M. persicae* in many parts of the world (Table 1). In tropical and warm temperate regions *M. persicae* is permanently thelytokous², but survival of translocation heterozygotes in the sexual cycle of the aphid,

where a translocation would be expected to impose a severe genetic load³, has also been demonstrated⁴. Considerable resistance to organophosphorus insecticides is common in glasshouse populations of *M. persicae*⁵, and laboratory clones originating from glasshouses and isolated for such resistance are invariably translocated. A link between the translocation and organophosphorus resistance has been suspected, as this could provide the selective advantage which would explain the worldwide occurrence of translocation heterozygotes and their survival in the breeding system. However, no information has been available on the comparative resistance of translocated and normal aphids from most parts of the world. Populations with moderate organophosphorus resistance that appeared in the field on sugar beet in England in 1974 (ref. 6) had the normal karyotype, as also did a resistant clone originating from peaches in France in 1968. Now we report evidence that directly implicates the translocation in the organophosphorus resistance of *M. persicae*.

In Japan, 17 clones of *M. persicae* were tested for their resistance to malathion using a dry film method⁷, with 10 apterae per vial (4×2 cm diameter). Mortalities were recorded 24 h after treatment. The six clones of normal karyotype showed greatest susceptibility to malathion (Table 2). Eight clones with the A1,3 translocation, including a laboratory-reared triploid⁴, showed various degrees of resistance, and one of these had a resistance factor of more than 100. The remaining three clones were heterozygous for an autosome 3 dissociation, which could be the first step in the rearrangement⁴. These also showed different degrees of resistance to malathion.

In England, 100 F₁ clones were obtained from a cross between sexual females (oviparae) from a karyotypically normal, organophosphorus-susceptible clone ('U'), and males from a translocated, very resistant clone (GCRI-1). The latter clone, which was approximately 100 times as resistant to dimethoate as the susceptible parent (U), was derived from a population on chrysanthemums in a glasshouse at Littlehampton, Sussex, and is believed to be a widely distributed clone of *M. persicae* in glasshouses in southern England. Eggs were overwintered in a well-ventilated cage in insectary conditions, and the hatching fundatrices were matured on radish plants and transferred to excised potato leaves in individual cages when adult, to give rise to separate F₁ clones.

Table 1 Occurrence of A1,3 translocation heterozygotes in samples of *M. persicae* collected 1972–1977

Origin of samples and years of collection	No. of samples examined	No. of samples with translocation heterozygotes
Great Britain		
glasshouses (1974–77)	15	15
field (1972–76)	48	1
Rest of Europe		
glasshouses (1972–76)	5	4
field (1972–76)	6	0
Rest of World (field only)		
Japan (1974–76)	31	18
Hong Kong (1976)	1	1
Philippines (1975)	2	2
Australia (1975–77)	18	7
New Zealand (1975)	3	1
Kenya (1975)	7	5
Nigeria (1975)	1	0
Rhodesia (1975)	1	0
South Africa (1977)	1	1
Canada (1975)	2	1
California (1975–77)	6	5
Michigan (1975)	17	16
Ohio (1975)	2	1
Brazil (1975)	1	1
Chile (1976–77)	2	0

Table 2 Resistance to malathion in relation to karyotype of 17 clones of *M. persicae* in Japan

Clone	Origin	Karyotype	LD ₅₀ per (µg per vial)	Fiducial limit (<i>P</i> = 0.05)	Resistance factor
URY-0	Field, on <i>Raphanus</i>	Normal	0.58	0.56–0.61	1.00
KM602	Field, on <i>Prunus</i>	Normal	0.69	0.55–0.85	1.18
UM604	Field, on <i>Prunus</i>	Normal	0.70	0.65–0.75	1.20
14-1	Laboratory reared	Normal	0.71	0.65–0.78	1.22
25-1	Laboratory reared	Normal	0.85	0.82–0.90	1.46
622-2	Laboratory reared	Normal	1.19	1.09–1.30	2.05
JCG-1	Field, on <i>Brassica</i>	A3 dissociation	1.72	1.55–1.92	2.95
UMG7501	Field, on <i>Prunus</i>	A1,3 translocation	1.93	1.63–2.29	3.31
622-1	Laboratory reared	A1,3 translocation	2.00	1.81–2.20	3.43
SCG-11	Field, on <i>Brassica</i>	A3 dissociation	2.43	2.41–2.47	4.16
HHR-1	Field, on <i>Brassica</i>	A1,3 translocation	2.89	2.16–3.94	4.96
622-5	Laboratory reared	A1,3 translocation	3.11	2.85–3.41	5.34
521-11	Laboratory reared	Triploid, with A1, 3 translocation	3.84	3.10–4.76	6.59
SCG-1	Field, on <i>Brassica</i>	A1,3 translocation	5.67	4.45–7.23	9.73
521-9	Laboratory reared	A3 dissociation	9.24	6.59–13.02	15.85
JCR-1	Field, on <i>Brassica</i>	A1,3 translocation	9.24	6.58–12.92	15.86
URR-0	Field, on <i>Raphanus</i>	A1,3 translocation	60.19	47.02–77.04	103.24

Thirty-five of the 100 *F*₁ clones were heterozygous for the A1,3 translocation. The other 65 were all of normal karyotype. Deviation from an expected 50:50 ratio⁴ was perhaps due to a hatching delay in aphids carrying the translocation; the clones were set up in approximately chronological order as the fundatrices matured, and only nine of nos 1–50 were translocated, compared with 26 out of nos 51–100.

Starch gel electrophoretograms were prepared from whole-body homogenates of four or five individual adult virginoparae (total weight 1.9–2.1 mg) from each clone, and stained for esterases⁸. All clones were examined in either the third or fourth generation after the fundatrix. There is good genetic⁸ and biochemical¹⁰ evidence that organophosphorus resistance in *M. persicae* is linked with increased carboxylesterase (est-4) activity. All 65 *F*₁ clones which were karyotyped as normal showed negligible est-4 activity, as in the normal, susceptible parent clone U (Fig. 1). Twenty-eight of the 35 clones which had the A1,3 translocation showed increased est-4 activity, although some samples clearly had less est-4 than the translocated, resistant parent clone, GCRI-1. The remaining seven translocated *F*₁ clones had est-4 activity characteristic of susceptible aphids. When additional samples of these seven clones were tested, using homogenates of single aphids, it was found that there were some individuals in each clone with increased est-4 activity (Fig. 2). Further studies revealed that est-4 activity varied between individuals in several other translocated *F*₁ clones, although there were some, like the parent clone GCRI-1, with a stable, high level of est-4.

Thus the translocation and the resistance mechanism show complete linkage, at least when inheritance is through the male. Spermatogenesis may normally be achiasmatic in *M. persicae*, as seems to be the case in two species of *Eucera*¹¹. Alternatively, the translocation may maintain its association with resistance by inhibiting crossing over. The question remains as to whether crossover suppression alone is sufficient to explain the frequency of the translocation in natural populations of *M. persicae*. It seems more probable that the translocation results in an advantageous rearrangement of genes contributing to the resistance mechanism. Preliminary studies of inheritance of organophosphorus resistance in *M. persicae* clones without the translocation indicate that at least two alleles are concerned in the regulation of est-4 activity⁹, although it is not known how many loci are involved. The translocation may bring resistance-associated genes which were previously on autosomes 1 and 3 into the same linkage group.

Linkage would be an advantage in itself, but there is some evidence that the chromosomal rearrangement actually enhances resistance by directly affecting gene action or interaction. First, *M. persicae* clones with the greatest organophosphorus resistance and est-4 activity, when examined cytologically, have invariably been translocated. Clone GCRI-1, with greater than 100-fold resistance, is nevertheless only heterozygous for genes conferring high est-4 activity. Second, the resistance of Japanese clones of *M. persicae* with the autosome 3 dissociation could be due to the inactivation of a regulatory locus on this chromosome. Third, the type of intraclonal variation illustrated by Fig. 2 is suggestive of a variegated (V-type) position effect¹²,

Fig. 1 Electrophoretogram showing linkage between a chromosomal translocation and increased est-4 activity in *M. persicae*. Parent clones GCRI-1 and U are on the extreme left and right of the gel respectively. In between are 16 representative *F*₁ clones, the eight to the left being translocated and the eight to the right being of normal karyotype. Each sample is the homogenate of four or five aphids. Starch gel, stained with Fast Blue BB salt using 1-naphthyl acetate as substrate.

EST 4 →

START →

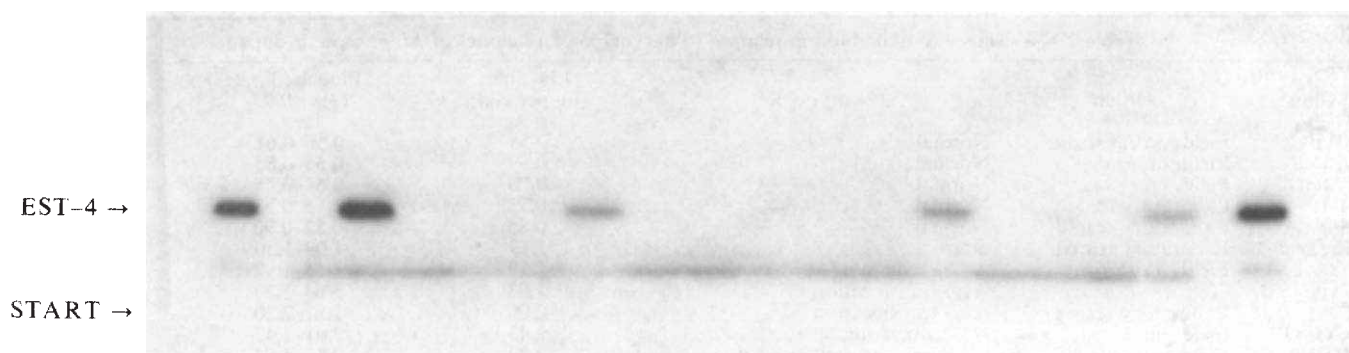


Fig. 2 Electrophoretogram showing variability of gene expression at the *est-4* locus between 16 individuals in one F_1 clone (TN 74) of *M. persicae*. Each sample is the homogenate of a single adult aphid. Individuals of the parent clone GCRI-1 are placed at both ends of the gel for comparison. Starch gel, stained with Fast Blue BB salt using l-naphthyl acetate as substrate.

such as might occur if resistance-associated loci were close to the point of translocation, and thus liable to inactivation by repositioned heterochromatin. V-type position effects classically explain variations in gene expression between cells within an individual, but could apply equally to differences between individuals within a clone. The extent of variegation in any one clone would be subject to the influences of modifiers in the genetic background, so that in different F_1 clones the expression of genes subject to position effect could vary either within individuals or between individuals in a single generation, or changes could occur over many generations. Intraclonal variation due to V-type effects would clearly complicate the results of insecticide bioassays, and might explain the widely different LD_{50} values obtained from translocated clones in the resistance tests in Japan (Table 2). It could also explain the instability of organophosphorus resistance within certain clones of *M. persicae*^{8,13-15}.

Further work is necessary to substantiate the position effect hypothesis. The variable activity of the *est-4* locus in translocated clones of *M. persicae* could prove a useful tool for studying certain aspects of gene expression.

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Does carcinogenic potency correlate with mutagenic potency in the Ames assay?

AN approximately linear relationship between the mutagenic potency of a chemical in the *Salmonella* reverse mutation assay of Ames¹ and its carcinogenic potency in animals, was recently alluded to² and has since been discussed at several international symposia^{3,4}. The implications of such a claim are clearly momentous. At present, the fact that the *Salmonella* assay can give both false positive and false negative results when compared with animal tests^{6,7} has led to its being regarded as an early warning system rather than the final arbiter of animal and possibly human carcinogenicity. A correlation between mutagenic and carcinogenic potency would, however, mean that a strong positive response in the Ames assay would define a potentially potent carcinogen, and would suggest that some compounds give positive results in this assay but fail to induce cancer in animals because their mutagenic potency is too low. We believe, however, that there is sufficient experimental evidence to treat any superficial potency correlation with great caution at this stage.

Our reservations about this correlation are based on the following. The concept that it is the absorption, distribution, metabolic activation and resultant half life of derived active species, rather than the *in vitro* mutagenic potency of a compound which determines its carcinogenic potency has been discussed by others^{8,9}. Also, some compounds are known to display species-dependent biological (including carcinogenic) properties¹⁰, yet it is from such species-dependent carcinogenicity studies that a potency correlation would have to be derived. Thus, a compound may be potentially carcinogenic as defined by an *in vitro* test yet be non-carcinogenic in the particular strain of species of animal in which it has been evaluated.

Apart from the above concerns in *in vivo* animal studies, serious doubts can be raised about the implied absolute quantitative significance of a *Salmonella* assay revertant colony count. In most cases, compounds which are mutagenic and/or carcinogenic require metabolic activation before they can elicit their effect. The *in vitro* medium usually employed for this activation is a sub-fraction of an homogenate of induced rat liver, the S9 microsomal mix. Despite its efficiency in a general sense (activation against no activation), the results mediated by this preparation are not generally regarded as being quantitatively significant. Also, although the general method of preparation and use of the S9 mix has been described¹, individual variations in protocol detail are common¹¹. If the assay results mediated by an enzyme preparation are to be quantitatively assessed then the enzyme preparation itself should first be calibrated in terms of the presence and activity of at least some of the basic activation and deactivation enzymes.

This is rarely, if ever, undertaken. Similarly, liver enzyme inducing agents such as Aroclor 1254, phenobarbitone or methylcholanthrene are used specifically to increase the levels or activity of certain enzymes, but in general no attempt is made to either quantitate this increase or to assess if it invariably leads to an increased mutagenic response.

The quantitative problems posed by unquantified and uncalibrated enzyme preparations can be illustrated by several recent studies. The carcinogen benz(a)pyrene (Fig. 1, I) requires metabolic transformation before it can elicit a carcinogenic or mutagenic effect. Although initial studies implicated the 4,5-epoxide (Fig. 1, II) (the K-region epoxide) as the critical intermediate^{12,13}, recent studies indicate that it is probably the 7,8-diol-9,10-epoxide derivative (Fig. 1, III) which is the important chemical species in cancer induction^{12,14-16}. The 7,8-diol group itself probably arises by attack on an intermediate 7,8-epoxide by epoxide hydratase enzymes¹⁴. It is, therefore, clear that the S9 mix used to activate benz(a)pyrene in an *in vitro* assay will be required to possess both oxidative enzymes for epoxide formation (such as cytochrome P-450) and epoxide-destroying enzymes, such as epoxide hydratase. In addition, various glutathione-S-transferase enzymes will be present in the S-9 mix and may react with any epoxide groupings present. It should therefore be possible to manipulate the muta-

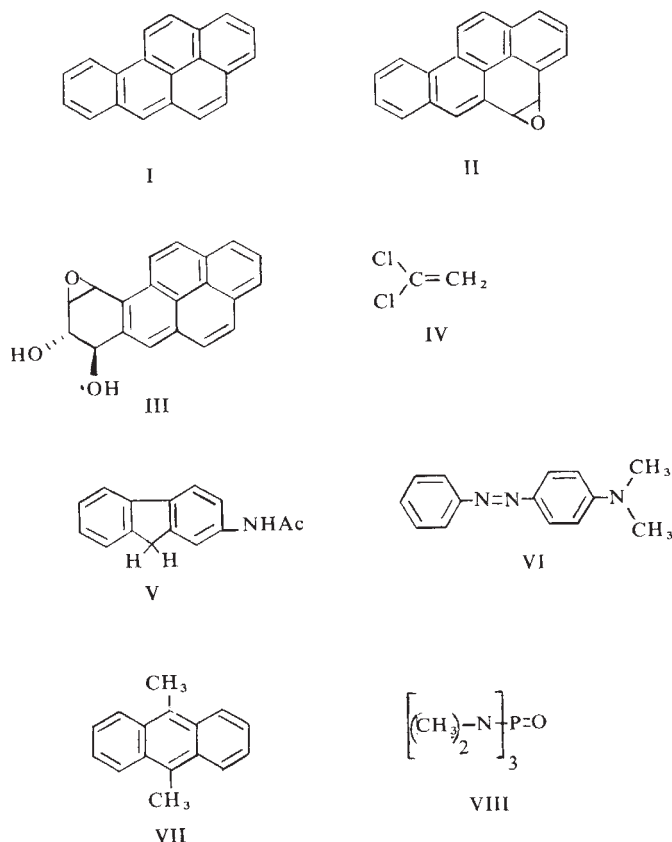


Fig. 1 Structures of test compounds.

genic response obtained for benz(a)pyrene in an *in vitro* test simply by varying the relative levels or activities of these three enzymes, and this is observed. Figure 2 shows the results obtained by Oesch *et al.* for benz(a)pyrene in the Ames assay^{17,18}. As expected, the addition of pure epoxide hydratase enzyme essentially abolished the mutagenic response while the chemical inhibition of this enzyme greatly enhanced the observed mutagenic response. Two factors are important here; first, both of the relevant enzymes are present but generally unquantified in the rat liver S9 preparations used for routine *in vitro* testing. Unsuspected variations in their levels or activities could dramatically alter the mutagenic potency observed for a compound competitively using these enzymes. Second, compound III is a poor substrate

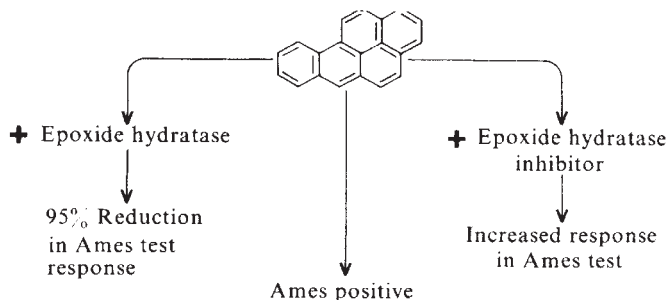
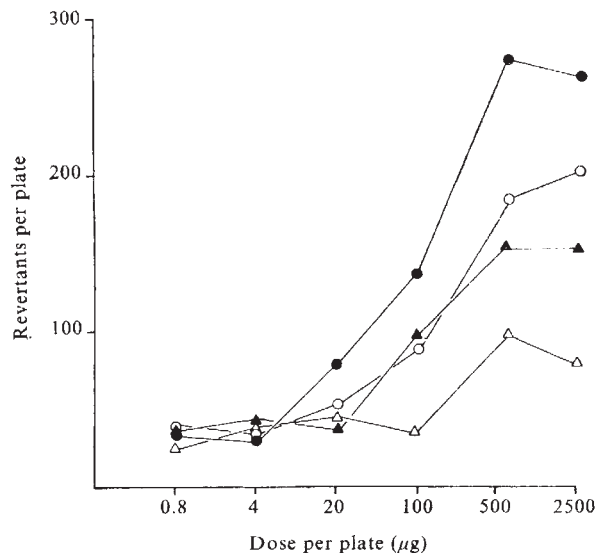


Fig. 2 Benz(a)pyrene action in Ames assay. Adapted from Glatt *et al.*¹⁷ and Oesch *et al.*¹⁸.

for epoxide hydratase¹⁴, which means that the mutagenic potency variations observed by Oesch were probably arbitrarily associated with the K-region epoxide¹⁹. A study of a series of 37 polycyclic aromatic hydrocarbon carcinogens²⁰ also revealed the overall lack of a correlation between carcinogenic potency and mutagenic potency in the Ames assay for that particular series of compounds. Similar findings have also been obtained for a series of structurally related nitrosamines²¹, while Bartsch has reported that vinylidene dichloride (Fig. 1, IV) is mutagenic in the Ames assay when using mouse derived S9 but almost inactive when using rat derived S9²². In the latter situation it is important to consider which result would be the relevant one to quote quantitatively *vis-à-vis* any possible animal carcinogenicity data. Further, the possibility that rats may possess the ability to detoxify epoxides more efficiently than do mice may account for the fact that trichloroethylene produces tumours in mice but not in rats²⁰ (the fact that the carcinogenicity of trichloroethylene has been suggested to arise from the butene oxide stabiliser present in the material tested²⁴ does not alter this argument).

This raises the question as to whether potency correlations should be applied within a given species of animal (for example, comparing the mutagenic potency of a compound in the Ames assay using hamster derived S9, with carcinogenicity data also derived in hamsters). The dangers inherent in this practice have been vividly demonstrated by McGregor, who showed²⁵ that cotton rat derived S9 was the best of eight different liver preparations, variously derived from five different species of animal, for the mutagenic activation of acetylaminofluorene (Fig. 1, V) in the Ames assay. Nonetheless, the cotton rat is

Fig. 3 Mutagenic response of *S. typhimurium* TA 1538 to benz(a)pyrene using Aroclor 1254 induced (○) and uninduced (●) mouse liver S9 mix and Aroclor 1254 induced (△) and uninduced (▲) rat liver S9 mix. Bacteria were obtained from B. N. Ames. Induction and preparation of S9 mix according to Ames *et al.*¹. The experimental protocol followed in all of these experiments is the same as in our earlier studies⁷.



claimed to be resistant to acetylaminofluorene-induced cancer²⁵. A further consideration is the diet employed during a carcinogenicity study or in animals from which livers are to be obtained for an S9 homogenate. The induction of liver tumours in rats with butter yellow (Fig. 1, VI) is dependent on the levels of the deactivating azoreductase enzymes in their livers. By reducing riboflavin intake in the diet, the levels of the riboflavin-dependent azoreductase enzymes are lowered, with a corresponding increase in tumour yield (that is, an apparent increase in carcinogenic potency)²⁶. It would therefore be expected that in the S9 mix used to evaluate related azo-compounds in the Ames assay, the levels of these enzymes would be both variable (by design or by chance) and critical to the apparent mutagenic potency observed. Similar nutritionally induced variations in liver cytochrome P-450 levels, with a resultant variation in enzyme-mediated compound metabolism, have also been reported¹⁰.

In an attempt to demonstrate clearly the dangers of detailed extrapolation of data generated in an *in vitro* test to the possible *in vivo* situation, we conducted a series of experiments with benz(a)pyrene. Five separate S9 fractions were prepared from uninduced and induced rat liver (Sprague-Dawley, Alderley Park strain), induced and uninduced mouse liver (Alderley Park strain, albino) and uninduced guinea pig. The results obtained in the Ames assay using these S9 fractions are shown in Figs 3 and 4. The guinea pig S9 fraction is incapable of effectively activating benz(a)pyrene into a mutagen while both the rat and mouse liver S9 fractions are variously effective. Thus, in this case of benz(a)pyrene at least, induction of the mouse and rat liver enzymes resulted in a reduction rather than an increase in the observed mutagenic potency.

In the supernatant of the S9 fractions there are glutathione-S-transferase enzymes and other chemical species which could react with epoxide intermediates and thereby inactivate them as mutagens. To investigate this possibility we removed the supernatant material from the guinea pig S9 fraction, leaving a pure microsomal fraction (105,000g fraction), and retested benz(a)pyrene using these microsomes in place of the original S9 fraction. The resultant positive effect is shown in Fig. 5. It can be inferred that the supernatant material of the guinea pig S9 fraction contained epoxide-destroying species which had been masking the mutagenic effect of any microsomally derived epoxides. Finally, the same guinea pig supernatant material was added to the microsomes (105,000g fraction) derived from the initial mouse S9 fraction and the reconstituted mouse/

Fig. 4 Mutagenic response of *S. typhimurium* TA 1538 to benz(a)pyrene using S9 mix made from uninduced mouse liver (Δ) and uninduced guinea pig liver ($\bullet$).

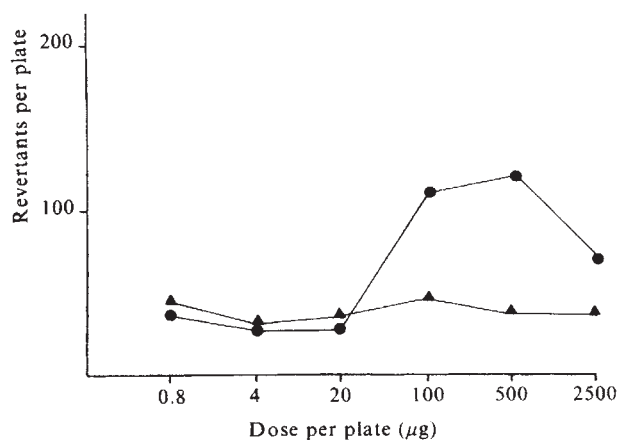
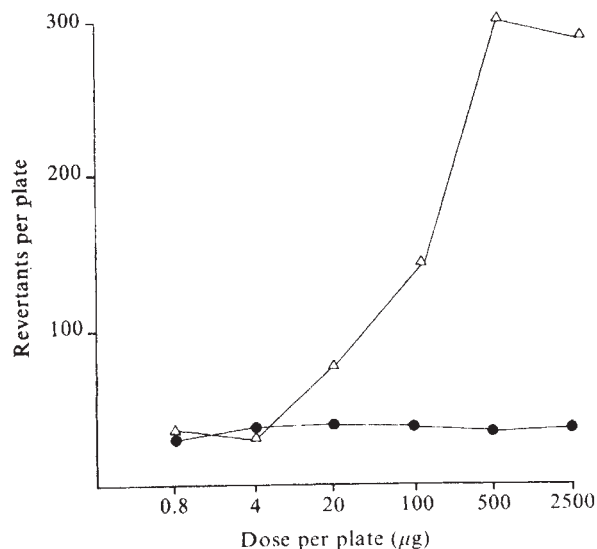


Fig. 5 Mutagenic response of *S. typhimurium* TA 1538 to benz(a)pyrene using guinea pig liver microsomes (105,000g fraction) with added cofactors ($\bullet$) and uninduced mouse liver microsomes (105,000g fraction) with supernatant from guinea pig (30,000g fraction) and co-factors ($\blacktriangle$).

guinea pig S9 fraction used to re-evaluate benz(a)pyrene. As expected, a negative response was obtained (Fig. 5). Thus, by simple fractionation of the S9 mix, the initial guinea pig negative response and the initial mouse positive response for benz(a)pyrene were both capable of reversal. These results, which are admittedly somewhat contrived in terms of the S9 preparations used for their generation, cannot be discounted as irrelevant because any metabolic activation system is presently regarded as acceptable as long as it can produce a positive result for a chemical.

The above experiments demonstrate that both mouse and guinea pig microsomes are potentially able to activate benz(a)pyrene into a mutagen, but that in a routine assay the observed result is strongly influenced by the surroundings in which the activation occurs, in this case the supernatant fluid. In the context of such sensitivity it should be anticipated that significant variation will occur in any biological parameter, such as mutation or cancer induction, when *in vivo* and *in vitro* responses are compared. A new dimension has recently been added to this debate by the co-mutagenicity effects described by Sugimura *et al.*²⁷⁻²⁹. By testing carcinogens such as butter yellow (Fig. 1, VI) in the presence of the non-mutagens harman or norharman, the mutagenic potency observed for the carcinogen can be dramatically increased (up to 40-fold in one case)²⁸. Further, compounds such as aniline and *ortho*-toluidine showed mutagenic properties under these conditions while being negative in the normal Ames assay²⁹. Whatever the explanation for these observations, their effect on an apparent potency correlation could be critical.

The variations in observed mutagenic potency of a chemical in the Ames assay discussed above are in general limited within a 100-fold range, which may seem negligible to an assay which has a 10⁶-fold mutagenic potency range². In our laboratory, however, this assay only has a 10³-fold range of mutagenic potency⁷. The above mentioned variations are, therefore, highly significant. There are also a sufficient number of exceptions to a potency correlation to discourage its rapid acceptance. For example, the very weak carcinogen 9,10-dimethylantracene (Fig. 1, VII) (ref. 30) is a potent mutagen in the Ames assay⁷. In contrast, the potent rat carcinogen hexamethylphosphoramide (Fig. 1, VIII) (ref. 31) is usually negative in this assay³², but is mutagenic as evidenced by a positive response given by this compound in *Drosophila*³³. The evaluation of potency correlations may be important when attempting to understand the mode of action and tissue or species specificity of individual carcinogens and mutagens^{34,35}. The premature acceptance of

an invariable correlation, however, may well reduce or at least impede the ultimate acceptance and beneficial impact of the Ames and related assays.

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Table 1 Mutagenicity of some compounds for *S. typhimurium* with and without S-9 mix

Compound	Amount per plate (µg)	No. of TA100 revertants S-9—	S-9+
Controls	0	226.3 ± 19.5*	204.6 ± 16.3*
2-Aminofluorene	1.25	237	585
	2.5	215	840
	5	242	1,075
	10	229	1,320
Sodium dichromate	10	312	198
	20	530	209
	40	825	231
	80	1,230†	980
Sodium azide	0.625	324	226
	1.25	710	284
	2.5	1,240	530
	5	2,270	1,085
Sodium nitrite	2,500	439	219
	5,000	585	373
	10,000	770	518
Captan	1.25	325	223
	2.5	397	284
	5	570	421
	10	1,115	645
5-Nitro-2-furoic acid	12.5	332	218
	25	845	307
	50	1,380	615
	100	384†	990

*Mean ± s.d.

†Toxic effects (small colonies and spare background lawn).

serial dilutions of compounds were made in water (sodium azide, dichromate and nitrite, and 5-nitro-2-furoic acid) or in dimethylsulphoxide (captan and 2-aminofluorene) and assayed by the plate incorporation test described by Ames *et al.*⁸. TA100 was used as bacterial tester strain. All assays were carried out in the absence and in the presence of the liver microsomal fraction (S-9 fraction) prepared from rats induced with a polychlorinated biphenyl mixture (Aroclor 1254, Monsanto), mixed in various amounts (10-50 µl per plate) with a fixed amount (0.5 ml per plate) of a NADPH-generating system (S-9 mix).

As shown in Table 1, 2-aminofluorene was mutagenic for strain TA100 of *S. typhimurium*, but only after addition of the liver microsomal preparation, while the other compounds were mutagenic *per se*, with dose-response effects. However, addition of the S-9 mix, containing 50 µl of S-9 fraction, resulted in a significant decrease of revertant colonies. The loss of mutagenic response was complete when test compounds were given at low doses, while larger amounts exceeded the deactivating capacity of the S-9 mixture used, with the exception of sodium

Table 2 Relationship between the amount of S-9 fraction embedded in the soft agar overlay and the number of TA100 revertants induced by mutagenic compounds

Compound	Amount per plate (µg)	Amount of S-9 fraction per plate (µl)					
		0	10	20	30	40	50
Controls	0	234	241	215	223	218	209
2-Aminofluorene	20	216	325	810	1,430	1,725	2,095
Sodium dichromate	40	705	420	370	283	228	221
Sodium azide	1	645	397	302	251	234	237
Sodium nitrite	5,000	568	454	396	361	358	341
Captan	2	845	570	436	390	327	296
5-Nitro-2-furoic acid	20	1,035	640	529	451	385	337

Metabolic deactivation of mutagens in the *Salmonella*-microsome test

THE *Salmonella typhimurium*/mammalian microsome test of Ames *et al.*¹ has provided a simple and sensitive short term assay for the detection of environmental mutagens. Moreover, there is excellent correlation between a positive reaction of the compounds so far tested in this prokaryotic test system and their carcinogenicity²⁻⁴. Metabolic activation of precarcinogens is achieved by incubating, on Petri dishes, the compounds to be tested, the bacterial strains and liver homogenates together with a NADPH generating system¹. The opposite phenomenon, that is conversion from mutagenically active to inert compounds, is also widely known to occur *in vivo*^{5,6} and inactivating enzymes have also been identified for some reactive metabolites⁷. However, the possibility of checking deactivation of the mutagenic potential of chemicals by means of the same *in vitro* system set up for metabolic activation has received little attention. The results reported here provide evidence that some compounds which are mutagenic *per se* for *S. typhimurium* can be partially or totally deactivated in the presence of liver homogenates. This possibility should be considered when results of the *Salmonella*/microsome assay are interpreted, and correlated with animal and epidemiological data.

The compounds tested were sodium azide, sodium dichromate and sodium nitrite (Merck), 5-nitro-2-furoic acid (Ega-Chemie), and captan (*N*-trichloromethylthio-tetrahydro-phtalimid) (Serva). 2-Aminofluorene (Ega-Chemie) was tested to check the activity of the rat microsomal preparation used. Solutions and

dichromate, which was totally deactivated below toxic concentrations. The metabolic deactivation of the compounds tested, as well as the metabolic activation of 2-aminofluorene, was correlated with the amounts of liver microsomal fraction embedded in the soft agar overlay (Table 2). Conversely, the number of revertants was not significantly affected by addition of S-9 cofactors without microsomal fraction.

Of the compounds studied, sodium azide, sodium nitrite, captan and 5-nitro-2-furoic acid had been included in the group of 'false' positive mutagens^{2,3}, that is of chemicals which were found to be mutagenic for *S. typhimurium* and gave negative or ambiguous results in animal tests. The reduction of the mutagenic activity in the *Salmonella* test system, in the presence of liver microsomal fractions, might account for the discrepancies between these *in vitro* and *in vivo* findings. Similarly, sodium dichromate and other hexavalent chromium compounds were associated with an increased incidence of lung cancer in man^{9,10} and were generally positive in animal assays, by inducing tumours at implant sites¹¹.

Further investigations are needed to elucidate the metabolic pathways leading to conversion of mutagenically active compounds into inactive metabolites. Use of microsomal fractions from various human or rat tissues should be helpful in assessing an appropriate correlation with epidemiological and animal data. The results of an extensive study of hexavalent chromium mutagenicity¹² suggest, for instance, that the metabolic deactivation of the metal by liver preparations is due to reduction to the trivalent form. In contrast to the hexavalent form, trivalent chromium is neither toxic nor mutagenic for *S. typhimurium*¹³ or *Escherichia coli*¹⁴. Interestingly, such conversion apparently also occurs in human erythrocytes, while rat lung and muscle microsomal fractions do not affect the mutagenicity of hexavalent chromium¹².

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Biochemical characterisation of stages of hepatocarcinogenesis after a single dose of diethylnitrosamine

CURRENT understanding of the pathogenesis of neoplasia rests principally on the demonstration¹⁻³ that the induction of carcinomas of mouse skin by hydrocarbons can be separated into at least two stages. There is evidence of similar stages in the natural history of carcinogenesis of nonepidermal tissues⁴⁻⁷. For example, Peraino *et al.*⁸ have shown that short term feeding of acetylaminofluorene to rats followed by a long term diet containing 0.05% phenobarbital results in 100% incidence of hepatomas, whereas animals receiving acetylaminofluorene and no phenobarbital developed 10 times fewer hepatocarcinomas. Peraino's procedure and that of Kitagawa *et al.*², who fed rats azo dye followed by phenobarbital, required that the

Table 1 Effect of phenobarbital fed to rats given diethylnitrosamine after partial hepatectomy

Treatment	No. of enzyme-altered foci per cm ³ liver at 8 months	Hepatomas
0.05% Phenobarbital (5)	0	0
DEN + PH (6)	3,370 ± 200	0
DEN + PH + 0.05% phenobarbital (8)	18,500 ± 900	7

Rats were partially hepatectomised (PH) and diethylnitrosamine (10 mg per kg) was administered as before⁸. Two months later one group receiving diethylnitrosamine and a control group were fed 0.05% phenobarbital with standard laboratory chow for a subsequent 6 months. The third group continued to receive the chow diet for the remainder of the experiment. At the end of this time animals were killed and sections of the liver were removed and frozen on solid CO₂. Serial sections of the frozen blocks of hepatic tissue were cut and stained consecutively with haematoxylin and eosin and for glucose-6-phosphatase¹⁰, canalicular ATPase⁹ and γ -glutamyl transpeptidase¹². Photographs were taken of each stained section and the number of enzyme-altered foci on each section was determined from tracings using transparent plastic. By appropriate overlaying of the three transparencies, one for each stain, the total number of enzyme altered foci could be calculated by the procedure of Scherer *et al.*²⁰.

carcinogen be fed to the rats for 3-8 weeks. On the other hand, Scherer and Emmelot⁸ have shown that a single large dose of diethylnitrosamine given to rats within 24 h of partial hepatectomy can induce hepatocellular carcinomas, whereas low doses (< 30 mg kg⁻¹), given in the same way, give rise only to small foci of cells deficient in ATPase, comparable with those described earlier^{9,10}. Further, Solt and Farber¹¹ have reported that a single dose of diethylnitrosamine followed by acetylaminofluorene and partial hepatectomy rapidly produces foci containing γ -glutamyl transpeptidase, in contrast to the situation in normal liver which exhibits no histochemical activity of that enzyme. By combining the procedure of Scherer and Emmelot⁸ with that of Peraino *et al.*⁸, we have been able to distinguish clearly between two stages in the genesis of liver cancer in rats.

We used the method of Scherer and Emmelot⁸ followed by the regimen of Peraino *et al.*⁸ (Fig. 1). The results are shown in Table 1. Unlike previous studies^{8,10}, ours involved the scoring of enzyme-altered foci in serial frozen sections by means of stains for three different enzymes: glucose-6-phosphatase¹⁰, canalicular ATPase⁹ and γ -glutamyl transpeptidase¹². In accord with the earlier studies^{8,11}, we found foci exhibiting a deficiency of glucose-6-phosphatase and/or canalicular ATPase as well as a positive stain for γ -glutamyl transpeptidase alone

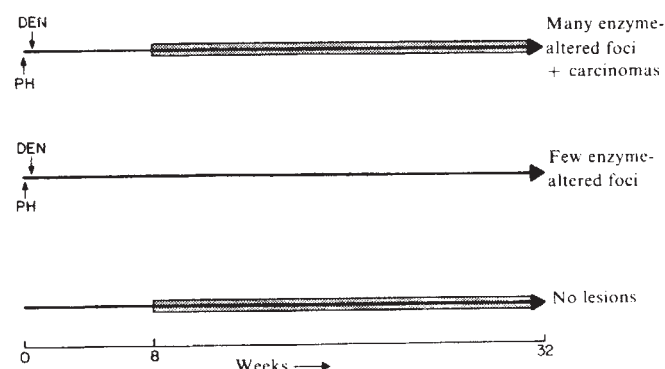


Fig. 1 Schematic representation of the protocol used for the induction of enzyme-altered foci and carcinomas in rats given a single dose of diethylnitrosamine (DEN, 10 mg per kg) by intubation 24 h after partial hepatectomy (PH)⁸. Eight weeks later a group of these animals was placed on a diet containing 0.05% phenobarbital (indicated by shading), while the other group received the same diet containing no drug. A control group was fed 0.05% phenobarbital during the same period. In all experiments female Sprague-Dawley rats weighing 200-220 g were used.

or in combination with a deficiency of one or both of glucose-6-phosphatase and ATPase (Table 2). Phenobarbital alone, even after 6 months, produced no enzyme-altered foci. But when administered for 6 months after a 2-month interval following partial hepatectomy plus diethylnitrosamine (5–10 mg per kg), this drug caused a six- to eightfold increase of enzyme-altered foci and most animals had hepatocellular carcinomas. Evidence that such enzyme-altered foci represent an early clonal stage in the development of hepatocellular carcinomas has been presented before^{9,13,14}, suggesting that in hepatocarcinogenesis it is possible to identify histochemically the immediate progeny of the putative initiated cells.

On the basis of earlier studies^{15,16}, we established the biochemical phenotype of each enzyme-altered focus using a suitable combination of overlay transparencies traced from photographs of three serial sections, each obtained for one of the enzymes in question. Figure 2 shows a typical composite overlay with different symbols for each area exhibiting one or more enzyme alteration(s). It was prepared from the liver of an animal given diethylnitrosamine (10 mg per kg) 24 h after partial hepatectomy followed by a 6-month dietary regimen of 0.05% phenobarbital. Extremely heterogeneous small foci and two larger foci of microscopic hepatocellular carcinomas can be seen. The phenotypic heterogeneity is analogous to that seen in both primary and transplanted hepatocellular carcinomas in rats¹⁵. Other studies have demonstrated that hepatocellular carcinomas are heterogeneous for both glucose-6-phosphatase¹⁷ and γ -glutamyl transpeptidase¹⁸.

By this technique, we counted the enzyme-altered foci of each of the seven possible phenotypes (Table 2). In animals given diethylnitrosamine alone, more than 75% of all foci exhibited only a single enzyme alteration with roughly equal frequency. In contrast, 45% of foci in the livers of animals given carcinogen followed by phenobarbital in their diet exhibited γ -glutamyl transpeptidase whereas 30% were equally distributed between those exhibiting ATPase deficiency alone and those exhibiting this deficiency in the presence of γ -glutamyl transpeptidase. The relative proportion of foci exhibiting defects of all three enzymes increased more than twice over that in the animals given the carcinogen only.

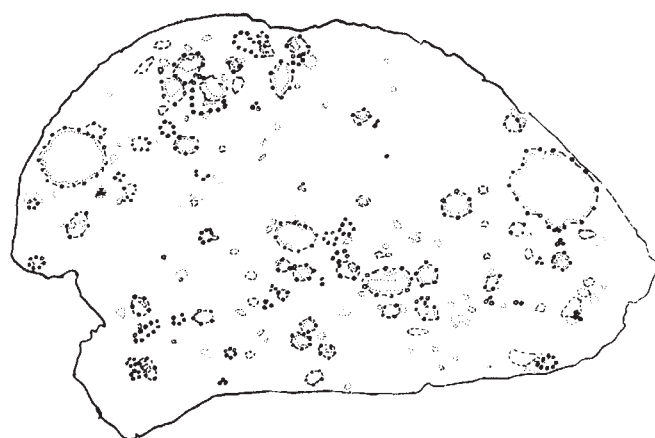


Fig. 2 Biochemical phenotypes of enzyme-altered foci in three serial sections of liver from a rat given a single dose (10 mg kg⁻¹) of diethylnitrosamine 24 h after partial hepatectomy followed 2 months later by feeding 0.05% phenobarbital for a subsequent 6 months. Each area is outlined to represent the enzyme alteration exhibited in that region as follows: 0–0–0, glucose-6-phosphatase-deficient areas; ----, ATPase-deficient area; γ -glutamyl transpeptidase present in area. In areas exhibiting more than one enzyme alteration, composite and/or parallel symbols are used. The figure was obtained by overlaying three separate transparencies after tracing on each of the outlines of areas exhibiting one enzyme alteration. Although in some areas not all cells in the focus exhibited the same phenotype, the area was scored as having the phenotype (Table 2) of most of the cells therein.

The mechanism by which the phenotypic heterogeneity of the foci is produced is not clear. In hepatocellular carcinomas this heterogeneity has been suggested to result from alterations in messenger RNA template stability¹⁵. If the phenotypic heterogeneity evident in enzyme-altered foci, the earliest discernible putative precursor of the hepatocellular carcinoma, reflects the phenotypes of the fully developed hepatocellular carcinoma, one may suggest that such biochemical changes

Table 2 Number and phenotypes of enzyme-altered foci per g of liver

	GP, AP, GT	GP, GT	GP, AP	AP, GT	AP	GT	GP	Total
DEN + PH	120±80 (3.6)	100±50 (3.0)	300±170 (8.9)	170±80 (5.0)	930±400 (27.6)	750±250 (22.3)	1,000±400 (29.7)	3,370±200 (100)
DEN + PH + 0.05% phenobarbital	1,450±300 (8.1)	300±100 (1.7)	700±250 (3.9)	2,700±700 (15.1)	2,700±700 (15.1)	8,700±1,100 (44.8)	2,000±500 (11.2)	18,550±900 (100)

GP, glucose-6-phosphatase negative; AP, canalicular ATPase negative; GT, γ -glutamyl transpeptidase positive; DEN, diethylnitrosamine; PH, partial hepatectomy.

Numbers in parentheses represent the percentage distribution of the foci in these conditions.

Five rats given DEN + PH alone and eight rats given DEN + PH + phenobarbital were used for these calculations. See text and Table 1 for further details.

These results show that two distinct stages can occur in hepatocarcinogenesis in analogy to that seen in skin. It is not clear how the promoting agent increases the number of enzyme altered foci; however, this system may be the first in which the immediate progeny of the putative initiated cells have been identified. Possibly, promoting agents not only cause the biological expression of malignancy but also stimulate the replication of dormant initiated cells, which in the absence of the promoter would not have proliferated. If each enzyme-altered focus is a clone derived from such a cell¹⁴, approximately one in 10⁴–10⁵ cells in the liver seem to be initiated by diethylnitrosamine as expressed after feeding with phenobarbital.

The production of enzyme altered foci in the liver may be a general result of hepatocarcinogen administration. Studies from our laboratory (unpublished) as well as those of Pugh and Goldfarb¹⁹ have demonstrated enzyme-altered foci following the administration of azo dyes and acetylaminofluorene to rats.

occur at or shortly after the process of initiation and remain relatively constant throughout the development of a malignant tumour from an initiated cell population.

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Myeloid colony-forming cells express human B lymphocyte antigens

STUDIES in experimental animals suggest that lymphoid and myeloid cells share a common stem-cell precursor¹, but human lymphoid-myeloid stem cells have been difficult to document. Indirect evidence for such cells has come from recent observations that human B lymphocyte antigens occur not only on B lymphocytes but also on monocytes and leukaemic myeloblasts²⁻⁶. If normal immature myeloid cells, like leukaemic myeloblasts, share a common differentiation antigen with B lymphocytes, it would seem even more likely that myeloid cells and B lymphocytes share a common stem-cell precursor. We have therefore compared the ability of bone marrow cells to form macrophage-granulocyte colonies *in vitro* after complement-mediated lysis of cells expressing either human B-lymphocyte antigens (HBLA) or human T-lymphocyte antigens (HTLA). Our results show that macrophage-granulocyte colony-forming cells (CFU-C) express HBLA but not HTLA antigens.

Ficoll-Hypaque separated mononuclear cells were obtained from bone marrows of children with acute leukaemia or lymphoma in remission. These cell suspensions were depleted of HBLA⁺ or HTLA⁺ cells by treatment with rabbit anti-HBLA or anti-HTLA plus complement (the preparation and specificity of these antisera have been described in detail previously^{5,7}). The viable cells remaining were incubated in methylcellulose with leukocyte-conditioned medium and examined for macrophage-granulocyte colonies after 10 d in culture.

The results of experiments using bone marrow cells from four different subjects are shown in Fig. 1. In every case, compared with control cells preincubated with normal rabbit serum + C, incubation of the marrow cells with anti-HBLA + C before culture resulted in a marked reduction in numbers of colonies formed on day 10. Incubation of cells with anti-HTLA + C, however, resulted, if anything, in an increase in colony numbers on day 10 of culture. Thus the myeloid precursor cells capable of forming macrophage-granulocyte colonies *in vitro* seem to express HBLA but not HTLA antigens. We have been unable to detect HBLA antigens by immunofluorescence or complement-dependent cytotoxicity on mature peripheral blood granulocytes (J.K., unpublished observations). These findings are consistent with recent observations by Ross *et al.*⁸ that rabbit antisera to purified lymphocyte membrane components detect B-lymphocyte antigens on normal bone marrow myeloblasts and myelocytes but not on metamyelocytes, band forms or mature granulocytes.

That B lymphocytes and immature myeloid cells share common antigens supports the concept that lymphoid and myeloid cells arise from a common stem-cell precursor. The fact, however, that T lymphocytes and even relatively im-

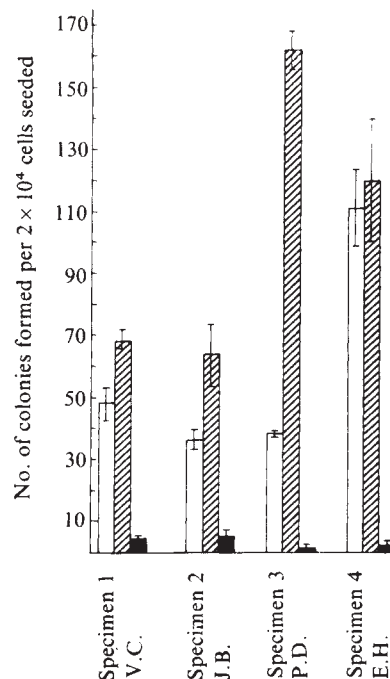


Fig. 1 Effect of complement-mediated lysis of HBLA⁺ or HTLA⁺ bone marrow cells on *in vitro* macrophage-granulocyte colony formation. Suspensions containing 10^7 cells ml^{-1} diluent (RPMI) 1640 (with 20% foetal calf serum) were incubated for 60 min at 37 °C with equal volumes of 1:10 diluted heat-inactivated rabbit anti-HTLA or anti-HBLA and complement (fresh normal rabbit serum). Control cells were incubated with heat-inactivated normal rabbit serum and complement. After determining the percentage viability by Trypan blue exclusion, the cells were washed three times and the cell concentration adjusted to 1×10^5 viable cells ml^{-1} alpha-media. The cells were cultured for 10 d in 1% methylcellulose in quadruplicate plates as described previously¹³ using leukocyte-conditioned medium at a 20% concentration (v/v) as the source of stimulators. Open columns, normal rabbit serum + complement; hatched columns, anti-HTLA + complement; solid columns, anti-HBLA + complement.

mature T-cell precursors⁸, lack HBLA antigens raises the question of whether the stem cells for T lymphocytes are distinct from the stem-cell precursors of B lymphocytes and myeloid cells. Recent observations that terminal transferase (TdT), an enzyme normally found only in thymocytes and a small population of immature bone marrow cells⁹⁻¹¹, occurs in HBLA⁺ as well as HTLA⁺ leukaemic lymphoblasts (J.K., unpublished data) and in some leukaemic myeloblasts¹² mitigates against this possibility and suggests instead that T cells, B cells and myeloid cells all share a TdT⁺ stem-cell precursor. From the present findings it would seem likely that such a stem cell also expresses HBLA antigens which are lost at a prethymic stage of T-cell differentiation, just as they are lost at a metamyelocyte stage of granulocyte differentiation. Attempts to test this model of leukopoiesis further would be facilitated by the availability of improved methods for the detection and isolation of human lymphoid-myeloid stem cells. Antisera to HBLA antigens could be useful tools in such endeavours.

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Stable variants affecting B cell alloantigens in human lymphoid cells

THE human major histocompatibility complex (MHC) is defined by four loci: *HLA-A*, *B* and *C* are serologically defined and *HLA-D* was initially defined by the mixed lymphocyte reaction (MLR)¹. There is now evidence that a group of HLA-linked, serologically defined B cell-specific alloantigens are the gene products of either the *D* locus itself or of closely linked genes^{2,3}. Strong associations are seen between alleles of *HLA-D* and the B cell-specific genotypes, and B cell antisera inhibit the MLR^{4,5}. The *D*-associated antigens differ from the products of *HLA-A*, *B* and *C* in chemical structure and in tissue distribution⁶. Human B-cell alloantigens resemble Ia antigens in mice. Analysis of the relationships among Ia antigens, MLC loci and other functions mapping in the I region is limited in both humans and mice by the availability of recombinant individuals⁷. Because such recombinants are rare, we have begun an analysis of this region by mutation⁸, using immunoselection to isolate mutants defective for MHC gene products in human lymphoid cell lines. We describe here the first Ia variants to be isolated and characterised in any species.

The B cell-specific alloantibodies used for selection of the variants were isolated from antiserum 0800 (Associated Bio-medical), which also contained antibodies against HLA-B8. This antiserum reacted with some non-B-8 cells when tested on B-cell lymphoid lines. UM21, a non-B-8 cell line which reacted with 0800, was used to absorb the non-HLA-8 antibody, which was then eluted from the cells by incubation for 30 min at 0 °C in glycine-HCl-saline buffer pH 3.0, 0.5 M. This material is referred to as 0800 eluate (0800E). 0800E has the properties of B cell antibody⁹: first, in individuals reactive with 0800E, 10-15% of PBLs were labelled by indirect immunofluorescence; second, after separation of lymphocyte subpopulations from reactive individuals by anti-F(ab) column or sheep red blood cell (SRBC) rosettes, the B cell-enriched fraction gave 60-65%

Table 1 2 × 2 Comparison of 0800 eluate and WIA1 antisera of 7th Workshop

	WIA1	
	+	-
0800 Eluate		
+	4	0
-	0	9

positive cells and the T-cell fraction <1% positive cells; third, fowl anti-β2 microglobulin, which blocks the cytotoxic activity of alloantisera to HLA-A, B and C but not of B cell-specific antibody¹⁰, reduced the titre of 4 A and B alloantisera on sensitive target cells, but did not alter the cytotoxicity of 0800E. The 0800E reacted with 4 of 17 B-cell lines and neither of 2 T-cell lines. Thirteen of the B-cell lines were also tested with 169 B-cell alloantisera of the 7th Histocompatibility Workshop (7WS) panel¹¹, and 0800E showed correlation with the antiserum cluster WIA1 (Table 1).

Table 2 Cytotoxicity titres of B cell-specific sera, HLA sera and anti-β2-microglobulin sera

	Wild type progenitor T5-1	Variants (6.1.6)	(8.1.6)
B cell-specific alloantisera			
0800 eluate	4	< 1	< 1
277	2	< 1	2
HLA-A and B alloantisera*			
HLA-B8			
0800 absorbed	16	8	16
CAMPBELL	4-8	4	4
CHAYRA	2	2	2-4
HLA-B27	16-32	16	16-32
HLA-A2 (R3 absorbed rabbit heterosera)	8-16	8	—
HLA-A1 SHILEY	4-8	4-8	8-16
B cell-specific heterosera			
814	128	8-16	128
Anti-55	64-128	4-8	—
Anti-23-30	400-800	< 1	400
R20 (anti-T5-1, absorbed with 6.1.6)	16-32	< 1	16-32
Anti-β2 microglobulin (rabbit)	256-512	256	—

All antisera were tested for cytotoxicity by the Terasaki method. Doubling dilutions were made in human AB sera (except for anti-β2-microglobulin where RPMI 1640 was used) and pooled rabbit complement which had been absorbed with T5-1 cells was used. The titre of a serum on a cell line is the highest dilution giving 50% kill.

*Alloantisera except where otherwise noted; HLA antisera for HLA-A2, B8 and B27 are described elsewhere¹².

The cell line used in the selection was T5-1, which has been used in previous studies on HLA variants¹². T5-1 has a pseudodiploid karyotype and HLA haplotypes *HLA-A1*, *B8* and *HLA-A2*, *B27*, *CW1*. With respect to B cell-specific alloantigens, the cell line reacts with all sera in clusters defining WIA1 and WIA3, which seem to be alleles of a single locus¹¹.

Before selection, T5-1 was mutagenised as described previously¹¹ to increase the frequency of variants. Logarithmically growing cells were suspended in either 4 μg ml⁻¹ ICR-191 or 50-100 μg ml⁻¹ ethylmethanesulphonate (EMS) for 24 h. After washing twice and resuspending in fresh medium, the cells were cultured until the viability was ≥80% as measured by Trypan blue exclusion. For the selection of variants, 2.5-5.0 × 10⁵ cells were incubated in 0.1 ml antiserum for 45 min at 22 °C, centrifuged and then exposed to pooled absorbed R C' in excess for 90 min at 37 °C, washed and then plated in soft agarose⁸. Surviving clones were isolated approximately 14 d later and tested with 0800E. Among a total 1 × 10⁶ cells treated with ICR-191 and selected against, a single 0800E-resistant clone was found (6.1.6). Another aliquot of the same ICR-191-treated cells gave 22 HLA-B27 variants for every 10⁶ cells selected. Two 0800E-resistant clones (8.1.6 and 8.2.6) were recovered from 2.5 × 10⁵ EMS-treated cells; another aliquot of these cells gave 24 B27 variants per 2.5 × 10⁵ cells.

Resistant clones 6.1.6 and 8.1.6 were tested with the selecting antiserum at least five times over a period of 6 months of cultivation in normal medium (a total of ≥80 population doublings) and the phenotype was stable. Cytotoxicity assays with a panel of A and B antisera (Table 2) showed that the variants were essentially unaltered in expression of HLA-1, 2, 27 and 8.

To define the nature of the antigenic loss further, the variants were tested with the 169 B cell-specific (Ia) 7WS alloantisera (Table 3) and with several rabbit sera prepared against partially purified human Ia. Variant 6.1.6 has lost reactivity with both WIA1 (4/4 sera) and WIA3 (5/5 sera) and, in fact showed no reactivity with any of the 44 sera that defined antigenic clusters. Of 58 sera that reacted with T5-1, only one weak reaction was retained, and this was with a serum which

also reacted with a T-cell line. In addition to the loss of alloantigenic determinants, 6.1.6 seems to have lost the constant portions of the Ia molecule as indicated by nonreactivity with anti-P23, 30 (ref. 6), a heteroantiserum prepared against papain solubilised, purified B cell-specific glycoprotein, which has a titre of 200–400 against T5-1. Antiserum 814¹³ and anti-55 (A. Fuks, unpublished) are antisera against immunogens prepared by detergent solubilisation and partial purification of the alloantigen-bearing glycoprotein dimer from two different B-cell lines and both showed reductions in titre (from 128 to 8–16 and from 64–128 to 4–8 respectively (Table 2)). In each case, this residual reactivity was approximately equal to that seen against the T-cell line HSB-2.

To demonstrate that the nonreactivity of 6.1.6. with anti-23-30 was not due to nonspecific masking of the antigen, deoxycholate was used to lyse cells and solubilise membrane glycoproteins from T5-1 and 6.1.6, and the lysates were tested for antibody-binding according to the method of Reif^{14,15}. In three experiments wild-type cells gave an average *g* value, that is, number of cells to absorb one half of cytotoxic antibody, equal to 2.1×10^3 cells/ λ , but in each experiment lysates from 6.1.6 bound no antibody at cell levels equivalent to 1.68×10^5 cells. Thus the amount of antigen in 6.1.6. is $< 1/80$ of that of the wild type.

Further evidence that 6.1.6 had undergone loss of the constant portion of Ia came from absorption experiments in which rabbit antiserum raised against T5-1 was absorbed with 6.1.6. The absorbed antiserum was tested for reactivity with cell lines and on B and T cells from peripheral blood (Table 4). This absorbed antiserum should be specific for determinants missing in the variant¹⁶. The absorbed antiserum reacted with T5-1 and with all B-lymphoid lines with approximately equal cytotoxicity titres but with neither of the two

T cell lines. Moreover, the absorbed antiserum reacted with titres of 8 to 16 on separated B lymphocytes from three individuals but it gave no cytotoxicity with the T lymphocytes. Thus heteroantiserum made against T5-1 and absorbed with 6.1.6 recognises determinants present on B cells of all 18 individuals tested but not on T cells, consistent with the interpretation that 6.1.6 had lost the common portion of Ia.

The variant 8.1.6, also selected as resistant to the 0800E, has a more limited lesion; it was indistinguishable from wild type with the heterosera described above. Tested with the 7WS panel, it showed loss of reactivity for WIA1 (4/4 sera) but unaltered reactivity for WIA3 (5/5 sera) (Table 3). The variant thus has an alteration in the WIA1 allele. Among the sera other than those mono- and multispecific sera recognising defined specificities, seven sera (our designation UW-X) react with T5-1 but not with 8.1.6. These 'UW-X' sera also react with WIA1 and WIA2 among our B-cell lines. The UW-X sera may represent polyspecific sera (which react only with IA-1 in T5-1) or they may detect a public specificity which is shared by IA1, -2, and -6 and which is included in the 8.1.6. lesion.

Table 4 Cytotoxicity titres of rabbit heterosera prepared against T5-1 (progenitor) and absorbed with variants

		Anti-T5-1	
		absorbed with 6.1.6	absorbed with 8.1.6
T5-1	10.2.5	16–32	4
	6.1.6	<1	—
	8.1.6	16–32	4
15 Unrelated B cell lymphoid lines*		4–8	—
T cell lines			
	8402	<1	—
	HSB-2	<1	—
Peripheral blood lymphocytes (3 donors)			
	B-cell fraction†	8–16	—
	T-cell fraction†	<1	—

Fixed volumes of antisera were absorbed with varying numbers of variant cells to attempt to find a minimal number which would remove all cytotoxicity against the variant. Data shown are for antisera absorbed with 1×10^6 cells λ^{-1} .

*The other B lymphoid lines tested were 8866, BREL, ORI, BRISTOL 8, UM-21, DAUDI, HUP-2, SB-1, SC-LA, SC-TA, SC-JB, SC-BM, SC-CA, SC-AM, RAJI, and two T-cell lines HSB-2, 8402.

†Peripheral blood lymphocytes were fractionated on an anti-F(ab) column. B-cell fractions contained 94–96% s Ig-positive cells and T-cell fractions contained 1–6 s Ig positive cells. — = not done.

The two variants, both selected with the same antiserum, thus have different properties from one another. This is consistent with evidence previously obtained^{11,17} that the antiserum selects pre-existing variants rather than inducing modulation of the reactive antigen. 8.1.6 seems to have lost the WIA1 determinant but has retained the WIA3 determinant as well as the 'common portion' of the molecule, as determined by reactivity with the heterosera. In this respect 8.1.6 seems analogous to most of the A and B variants previously isolated. 6.1.6 is altered not only for the WIA1 alloantigenic determinant selected against but also does not express the allelic determinant WIA3 nor any 'constant portion' of the HLA-linked B cell specific glycoprotein as judged by two independent lines of evidence; first, the absence of antibody-binding from anti-23, 30¹⁸ by 6.1.6 whole cells or lysates, and, second, rabbit anti-T5-1 absorbed with 6.1.6 reacts with all B cells tested but with none of the T cells. 6.1.6 could be explained by a single mutation in a regulatory locus or by mutations in two structural genes or by an epigenetic event. Although 6.1.6 lacks both WIA1 and WIA3, the phenotype is unlikely to be the result of two mutations, one in each of the genes carrying these determinants because the frequency of such an event would be the square of the single event and no single mutants were observed among the ICR-191-tested cells. If 6.1.6 is due to a change in a

Table 3 IA typing of T5-1 and variants by 7WS panel

WIA antigen	Serum	Cytotoxicity scores*		
		T5-1	6.1.6	8.1.6
1	7W009	3.5	—	—
	7W042	4.0	—	—
	7W120	4.0	—	—
	7W128	3.5	—	—
	7W152	4.0	—	—
3	7W024	3.5	—	4.0
	7W026	4.0	—	4.0
	7W028	4.0	—	4.0
	7W035	4.0	—	4.0
	7W133	2.0†	—	4.0
6+3	7W075	4.0	—	4.0
	7W111	4.0	—	4.0
	7W157	4.0	—	4.0
6+2(+1)	7W046	4.0	—	†3.0
	7W144	4.0	—	—
	7W179	3.0	—	—
UW-X	7W004	4.0	—	—
	7W010	4.0	—	—
	7W066	4.0	—	—
	7W105	4.0	—	—
	7W114	4.0	—	—
	7W150	3.0	—	—
	7W156	4.0	—	—

The sera of 7WS have been grouped into clusters defining eight groups. Results are shown for sera in clusters WIA1, WIA3, WIA6+3, WIA6+2+1 and a group of sera UW-X. Not shown are results for those clusters which gave a negative reaction pattern on both T5-1 and its variants. (Clusters WIA2, 4, 5, 7, 8, WIA A, IA4×7.) UW-X is the group of sera which react with T5-1 and are altered in the variant 8.1.6. (Analysis of UW-X on normal unrelated cell lines showed caucasian derived lines with either WIA1 or WIA2 were reactive; also reactive were two non-caucasian-derived lines, Daudi (African Black) and SJ-AH (Japanese), which are not WIA1 or WIA2. For discussion of nature of UW-X, see text.)

*Methods for cytotoxicity tests as in Table 2. Scores are means of two tests of undiluted antibody; values 2, 3, 4 indicate consistent positive reactions of increasing strength. —, Score ≤ 1.5 .

†Cases in which difference in score between replicates ≥ 2 .

regulatory locus, it is not associated with a switch to expression of a different alloantigenic allele detectable by alloantisera or by absorbed rabbit antisera. The possibility that 6.1.6 arose by an epigenetic event is an open one unlike previously described variants for HLA-A and B where there is a preponderance of evidence for the variants being the products of single gene mutations^{8,11,17}. Regardless of the nature of the 6.1.6 lesion, it is clear from the unchanged expression of HLA-A and B that it does not affect these other MHC-coded membrane glycoproteins.

The typing of alloantigenic variants such as 8.1.6, delineates Ia specificities in a manner which is independent from the conventional delineation by pedigree analysis. If the human Ia system resembles murine Ia in being composed of several tightly linked loci, the mutational approach should make this apparent by distinguishing the products of independent loci whereas pedigree analysis would require the discovery of rare recombinants. In the case of 8.1.6 the fact that reactions with five WIA1 sera were altered coordinately suggests that these sera probably are all reactive with the products of the same single locus. Ia variants should also be useful in exploring other questions such as the nature of the determinants which stimulate in the MLC and the regulation of Ia expression.

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Production *in vitro* of murine antibody to a human histocompatibility alloantigen

AN *in vitro* culture system from our laboratory which may be used to generate homogeneous antibodies to human cell surface antigens has been recently described¹. Using this system, we have obtained series of antibodies which discriminate between closely related human cells¹ and cell lines². The present report defines more precisely the potential of this system by showing how it may be used to obtain antibodies to a human cell surface antigen of well-defined specificity, the histocompatibility alloantigen HLA-A2.

The mouse spleen fragment culture system involves injecting limited numbers of donor spleen cells, with immunogen, into irradiated, syngeneic recipients; the recipient spleens are diced

into 1-mm³ fragments, which are cultured individually. The dose of donor cells is adjusted so that each fragment receives at most a few antibody-forming cell precursors which recognise the immunogen; these precursors proliferate during the culture period, and the antibody produced by the daughter cells is secreted into the culture medium¹⁻³. In the present study, the A2⁺ human lymphoblastoid cell line 8866 (ref. 4) was used as the immunogen, and the culture fluids from 167 fragments were studied. A radioimmunoassay^{2,5} was used to measure the antibody-binding activity of each fluid against both 8866 and an A2⁻ variant of that line, subline 1-2. This variant was isolated by immunoselection with an allogeneic anti-A2 antiserum, and lacks both cell surface and internal A2^{1,6,7}. We found that 141/167 fluids contained antibody to 8866, and all but three of these showed identical binding to that cell and its A2⁻ variant. The three remaining fluids, B9, F4 and H4, showed significantly greater binding to the A2⁺ cell (Fig. 1a-c).

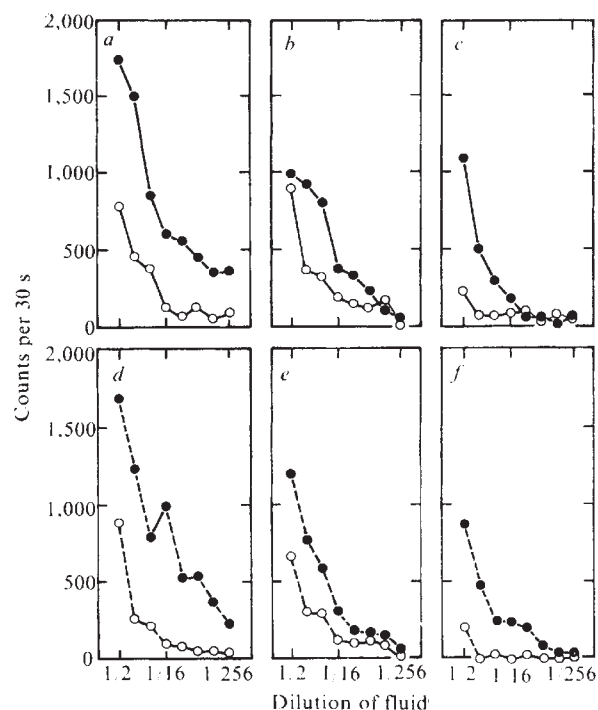


Fig. 1 Binding of discriminator culture fluids to A2⁺ and A2⁻ cell lines. Antibody binding activity in culture fluids was assessed by radioimmunoassay. Generation of culture fluids: 10⁷ viable donor spleen cells were given intravenously to each of four lethally irradiated recipient mice; a few minutes later, 10⁷ 8866 cells were given intravenously to each recipient; 18 h later, recipient spleens were diced into 1 mm³ fragments and the fragments were cultured individually in 96-well plastic plates for 4 weeks, during which time the culture fluids were collected and replaced with fresh medium every 3-4 d. Donor mice were (BALB/c × C57BL/6) F1 mice that had received intraperitoneal injections of 10⁷ 8866 cells 15, 9, and 6 weeks before use. Recipient mice were F1 mice that had received a single intraperitoneal injection of 10⁷ 8866 cells 5 weeks before use. Radioimmunoassay: 25 μ l of culture fluid was incubated for 2 h at room temperature with 2 times 10⁵ glutaraldehyde-fixed target cells; target cells were washed extensively, and then incubated with 10 μ l ¹²⁵I-goat anti-mouse κ (30,000 c.p.m./10 μ l) (ref. 2) for 18 h at 4 °C; the cell pellets were washed extensively, dried, and counted in a γ counter. Graphs show (counts bound with test fluid) - (mean of counts bound with eight control fluids). Control fluids were generated by culturing fragments from spleens which had received neither donor cells nor antigen. Mean control binding for each cell type: 8866, 188; 1-2, 214; T5-1, 211; 6.6.5, 202. a-c, Binding activity to 8866 (●) and variant 1-2 (○); d-f, binding activity to T5-1 (●) and variant 6.6.5 (○). Fluids F4 and B9: fluids collected after 7 and 10 d of culture were pooled for assay; a later collection (after 14 d) showed similar discrimination between A2⁺ and A2⁻ cell lines (not shown). Fluid H4: assays were performed on 14-d collection; an earlier collection also showed discrimination, but less overall activity (not shown). a and d, Fluid B9; b and e, fluid F4; c and f, fluid H4.

The specificity of the three discriminator fluids was verified in two ways; first, the radioimmunoassay was used to show that each of the three fluids also discriminated between a second A2⁺ cell line, T5-1, and an A2⁻ variant of that line, subline 6.6.5 (ref. 8) (Fig. 1d-f); second, a cytotoxic assay was used to assess the specificity of the fluids with respect to normal human peripheral blood lymphocytes (PBL). Each of the three fluids was cytotoxic for 2/2 A2⁺, A28⁻ PBL, and negative for 2/2 A2⁻, A28⁻ PBL (data not shown). Fluids B9 and F4 were also tested in a more extensive typing panel (Table 1), which confirmed that both fluids recognised A2⁺ cells, as well as cells bearing the highly cross-reactive⁹ specificity A28. Neither fluid

Table 1 Cytotoxic activity of discriminator fluids against normal human PBL

Test cells	Fluid B9		Fluid F4	
	Sample 1*	Sample 2†	Sample 1*	Sample 2†
A2 ⁺ , A28 ⁻	13/13	14/14	12/13	2/2
A2 ⁻ , A28 ⁺	6/7	12/12	5/7	2/2
A2 ⁻ , A28 ⁻	0/10‡	0/19§	0/10‡	0/2

Cytotoxic testing was performed in plastic typing trays, using PBL labelled with fluorescein diacetate (FDA): 2,500 cells in 1 µl were incubated with 1 µl of culture fluid for 30 min at room temperature, after which 5 µl of fresh frozen rabbit serum was added; cell viability was determined after an additional room temperature incubation for 2h 30 min. Table shows the ratio of target cells showing strong positive reactions to target cells tested.

*Fluids collected after 7 and 10 d of culture were pooled for assay.

†Assay performed on fluid collected after 14 d of culture.

‡All specificities known to be present on the immunising cell⁴ were represented.

§All known HLA-A and HLA-B specificities and most Aw and Bw specificities were represented.

was cytotoxic for any cell lacking both of these specificities; in particular, neither fluid recognised any of the other HLA specificities known to be present on the immunising cell⁴.

In order to test whether the A2 and A28 reactivities were separable by absorption, serial dilutions of fluids B9 and F4 were absorbed with constant numbers of each of three types of PBL: A2⁺ cells, A28⁺ cells and, as a control, A2⁻, A28⁻ cells. The residual cytotoxic activity against both A2⁺ and A28⁺ target cells was then measured (Table 2). For each fluid, absorption with a constant number of A2⁺ cells significantly reduced the cytotoxic activity against both A2⁺ and A28⁺ target cells. Similarly, absorption with A28⁺ cells significantly reduced the cytotoxic activity against both types of target cells. These results suggest that fluids B9 and F4 contain antibody which is directed against a cross-reactive determinant present on both A2⁺ and A28⁺ cells. Although the cross-reactivity between these cells is well established, its molecular basis is not yet fully understood⁹. Antibodies such as those described here should prove valuable tools in analysing these problems. Figure 1 shows that fluids B9 and F4 each showed some binding activity to the A2⁻ cell lines. Because both A2⁻ variants have been shown to lack surface A2 and A28 (refs 4 and 6, and D. P., unpublished results) we attribute this binding to the production of one or more additional antibodies by these fragments. Also, because no extra activities were seen in the extended typing panels, these antibodies may not recognise (or not be cytotoxic for) antigens found on normal human PBL. These points will be explored more fully in a later report (L. A. L. *et al.*, in preparation).

Many laboratories have produced xenogeneic anti-HLA antisera^{10,11}. Our approach differs from previous work in that murine anti-HLA antibodies were produced *in vitro*, and that, even though a whole cell was used as the immunogen, we obtained antibodies which, without further purification, were specific with respect to HLA. The value of *in vitro* cloning techniques for the production of homogeneous antibodies to cell

Table 2 Absorption of discriminator fluids

Absorbing cell	Cytotoxic titre remaining after absorption			
	Fluid B9*		Fluid F4†	
	Target cells A2 ⁺ , A28 ⁻	Target cells A2 ⁻ , A28 ⁺	Target cells A2 ⁺ , A28 ⁻	Target cells A2 ⁻ , A28 ⁺
B.C. (A2 ⁻ , A28 ⁻)	32	≥ 64	4	2
K.W. (A2 ⁻ , A28 ⁻)	—	—‡	4	2
J.S. (A2 ⁺ , A28 ⁻)	4	4	0	0
C.A. (A2 ⁺ , A28 ⁻)	4	4	1	1
B.S. (A2 ⁻ , A28 ⁺)	8	8	1	0
L.B. (A2 ⁻ , A28 ⁺)	8	8	1	0

One µl (45,000) absorbing cells were incubated with 1 µl culture fluid for 1 h at 37 °C, after which 1 µl (2,500) FDA-labelled target cells were added, and the cytotoxic test carried out as in Table 1.

*Day 14 collection. Pre-absorption titres: A2⁺, A2⁻ target, ≥ 64; A2⁻, A28⁺ target, ≥ 64.

†Day 7+10 collection. Pre-absorption titres: A2⁺, A2⁻ target, 4; A2⁻, A28⁺ target, not done.

‡Not done.

surface antigens is widely recognised^{1,2,12,13}. This report demonstrates the ability of the mouse to recognise a well-defined human cell-surface alloantigen, and describes conditions under which there is a reasonable probability of finding antibodies specific with respect to HLA. In addition, we have shown the value of using HLA⁻ variant cell lines⁸ for the rapid identification of these antibodies. The mouse spleen fragment culture system, as used here, provides a versatile way of obtaining antibodies to human cell surface antigens in quantities suitable for analytical work^{1-3,14}. This system also provides a way of enriching for antibody-forming cells of a desired specificity; these may then be expanded by techniques such as transfection or somatic cell hybridisation into a more permanent source of the desired antibody.

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Coupling PPD to tumour cells enhances their antigenicity in BCG-primed mice

STUDIES of cell cooperation and of the carrier effect in the induction of antibody formation and in delayed hypersensitivity¹ can predict, and have indeed shown²⁻⁴, that combining an antigenic determinant which evokes a powerful T cell response with a weak antigen should greatly enhance the immunogenicity of the latter by providing 'antigenic help'.

Tuberculin (purified protein derivative, PPD) is a particularly effective carrier antigen. Long lasting and powerful T-cell reactivity (or delayed hypersensitivity) to tuberculin can readily be induced by infection with *Mycobacterium tuberculosis* vaccine (BCG). On the other hand, PPD itself does not induce delayed hypersensitivity but elicits reactions in the sensitised host. This property allows the effects of 'antigenic help' to be distinguished from changes in immunogenicity produced by chemical modification of the antigen simply by comparing the effects in BCG-primed and normal animals. Furthermore, there is little, if any, antibody response to PPD itself. Previous work from our laboratory has shown that PPD acts as a powerful carrier for the raising of anti-NIP antibodies in BCG-positive but not in BCG-negative hosts⁵. Immunising BCG-positive animals with PPD coupled to a weak protein antigen (for example, $\beta 2$ microglobulin) similarly enhances markedly the amount of anti- $\beta 2$ microglobulin that can be made (P. J. Lachmann, unpublished observations). We here report on the use of chemical modification with tuberculin for producing 'antigenic help' in raising immunity to the tumour specific transplantation antigens (TSTAs) of methylcholanthrene-induced sarcomas in syngeneic mice.

The tumours used were induced in female C57B1/10 ScSn (B10) mice by the subcutaneous implantation of 0.5 μ g 3-methylcholanthrene and were selected for immunogenicity as previously described⁶. The two tumours used in this study (designated MC 6A and MC 6B) were produced in a single mouse and were found to be non-crossreactive in a rejection assay. In initial experiments, tuberculin (PPD, Central Veterinary Laboratories, Weybridge, Surrey) was coupled directly to the irradiated tumour cells using redistilled glutaraldehyde at a final concentration of 0.005 M for

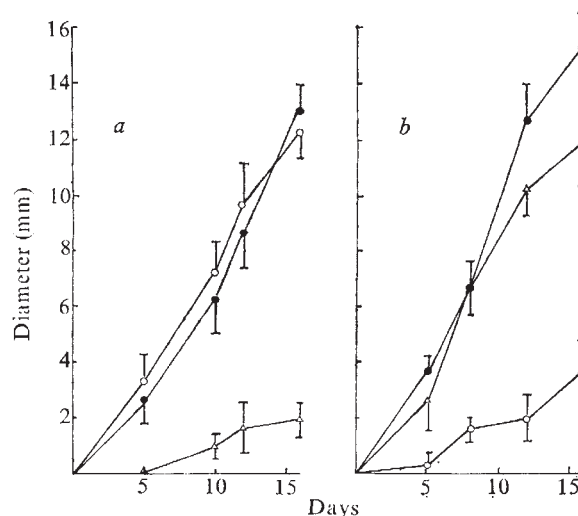


Fig. 1 Specificity of protection by cells coupled to Con A-PPD. Groups of three mice were immunised with either MC 6A (Δ) or MC 6B ($\circ$) cells coupled to PPD as in the legend to Table 1. Animals were challenged with 2×10^5 cells of either MC 6A (a) or MC 6B (b). Groups of unimmunised mice ($\bullet$) were similarly challenged. Mean tumour diameters were measured at 3-d intervals. Error bars represent $\pm$ s.e.m.

2 h at room temperature. This procedure, however, seemed to cause damage to the tumour specific antigens and for this reason an alternative strategy was employed. The tuberculin was coupled to concanavalin A (Con A) using glutaraldehyde as above. For this purpose 10 mg of Con A (Calbiochem) in 1 ml was mixed with 10 mg of 125 I trace-labelled PPD also in 1 ml and redistilled glutaraldehyde was added to a final concentration of 0.006 M. After 2 h at room temperature the reaction was stopped by the addition of 1/10 volume of molar lysine. The mixture was then fractionated by affinity chromatography on G75 Sephadex. The material passing through this column (more than 95% of the tuberculin added) was rejected and then Con A and Con A-PPD were eluted from the column with 0.1 M α -methylglucose. The Con A concentration in the eluted peak was measured by the agglutination titre on guinea pig erythrocytes and the PPD by counting the 125 I. This isolated Con A-PPD was dialysed to remove sugar and added to irradiated (10,000R) tumour cells (the equivalent of 20 μ g PPD was added to 10^8 cells). After incubation at room temperature for one hour, the cells were washed three times and the amount of bound 125 I determined by counting the washed cells in a γ counter; 25-50% of the added counts bound to the cells. The amount offered to the cells was chosen to give between 2 and 5×10^6 molecules of PPD per cell.

Groups of animals were immunised as described in Table 1. All the immunisations were performed on both BCG-primed and control (BCG-negative) animals to allow the role of delayed hypersensitivity to tuberculin to be assessed. Immunisation with tumour cells mixed with tuberculin was compared to immunisation with PPD-coupled cells. This allows the effect of injecting the cells into a site of delayed hypersensitivity reaction in the former case to be compared with 'antigenic help' in the latter. The effect of Con A coupling alone was also assessed.

From the results of the experiments as shown in Table 1 and Fig. 1 the following effects can be clearly seen: (1) powerful augmentation of immunity is seen in the test animals (group h) which are immunised so as to produce specific tuberculin help in immunisation (Table 1 and Fig. 1). It is of interest that the specificity of the rejection assays with regard to 6a and 6b is well maintained (Fig. 1); (2) the BCG status of the animal itself does not confer any

Table 1 Immunisation groups using Con A-PPD

Immunisation regime	Antigenic Ratio	
	Control (no BCG given)	BCG-primed
Irradiated cells only	a 1.7	b 1.5
Irradiated cells reacted with Con A	c 1.6	d 1.5
Irradiated cells mixed with PPD	e 1.4	f 1.0
Irradiated cells coupled to PPD	g 1.2	h 10.7

12-20-week-old C57B1/10 ScSn (B10) mice (OLAC, Bicester) were immunised with one tenth of the human intradermal dose of BCG (Glaxo, Greenford) in 0.2 ml saline by intraperitoneal injection. After two weeks, groups of three BCG-primed and untreated mice were injected with the following subcutaneously into the right groin in 0.2 ml saline: groups a and b: 10^7 radiated 6A cells, groups c and d: 10^7 6A cells reacted with Con A, groups e and f: 10^7 6A cells mixed with PPD, groups g and h: 10^7 cells coupled to PPD through Con A as described. A week later, all groups received 10^7 irradiated cells subcutaneously into the right groin as a booster immunisation. Ten days after this the mice were challenged with 2×10^5 live 6A cells injected subcutaneously in 0.1 saline also into the right groin. A group of immunised mice was also challenged. Tumour diameters were measured with calipers in two perpendicular planes and the mean taken. Results are expressed as an antigenic ratio: mean tumour diameter in unimmunised mice/mean tumour diameter in immunised mice. An antigenic ratio (A.R.) of greater than 1.0 indicates some immunity. The antigenic ratios shown are based on measurements on the sixteenth day after challenge.

increased immunity to the tumours (Table 1). If anything, the BCG-positive animals support tumour growth slightly better than do BCG-negative animals when uncoupled cells are used. In view of the widespread use of repeated BCG treatment in the immunotherapy of human tumours, it is worth pointing out that such repeated administration of BCG particularly at the tumour site is aimed at activating the macrophages, not at inducing tuberculin sensitivity; (3) there is no benefit to be seen from immunising with irradiated tumour cells into the site of the tuberculin reaction (Table 1). This is a manoeuvre that has in the past been claimed to give adjuvanticity⁷, but in the present model, benefit is not apparent; (4) Con A itself, when coupled to cells, produces no enhanced response (Table 1).

The rejection assay is believed to represent predominantly a cell-mediated reaction and therefore is likely to represent cooperation between the two sets of T cells. This potentiation effect clearly requires the 'carrier' molecule (tuberculin) to be coupled to the tumour cell rather than mixed with it and given into a pre-sensitised host. The conditions are thus typical of the carrier-hapten situation found in T cell-B cell cooperation, the PPD being the carrier and the tumour specific antigen, the hapten.

The tumours studied were selected for immunogenicity. Immunisation with three or more injections of irradiated tumour cells on their own is capable of producing a good degree of immunity. Further studies are in progress to see to what extent resistance can be achieved to tumours of lower degrees of immunogenicity.

The technique here described is of wide applicability, since Con A reacts well with nearly all cells and particularly with tumour cells, and the single Con A-PPD reagent can therefore be used for coupling PPD to different cells at will. Furthermore, since BCG has been frequently given without ill effect to humans with tumours, there would seem to be no ethical problem in extending studies of this kind to immunotherapy of human tumours.

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Host-donor cellular interactions in the treatment of experimental osteopetrosis

OSTEOPETROSIS is a rare bone disease of man, animals and birds¹. A striking feature is the failure of resorption of bone during growth and a reshaping of the skeleton so that the medullary cavities of shaft bones, normally containing only soft tissue—bone marrow—are filled with unabsorbed primary spongiosa causing a characteristic opaque radiological appearance that gives the disease its name. The osteoclast, a multinucleated giant cell, is considered to be responsible for the resorption of bone², and it has long been held that osteopetrosis is due to deficient osteoclasts. That the defect had a cellular rather than a humoral basis¹ was indicated when it was shown

that bony resorption in osteopetrotic mice was resumed after short periods in parabiosis with normal litter mates^{3,4} (which also showed that a circulating cell was involved) and after infusions of phenotypically normal and histocompatible haematopoietic cells^{5,6}. The subsequent and often rapid disappearance of the calcified material suggested to us that a new and proliferating population of cells was responsible, and to test this donor cells were tracked by a chromosome marker technique⁷. We now report that only low proportions of donor cells were located.

We used microphtalmic mice which suffer from osteopetrosis and were derived from Grüneberg⁸ (G) and bred at the MRC Radiobiology Unit, Harwell. The recessive *mi* gene in the homozygous state determines osteopetrosis. The *mi* gene was bred into the standard CBA/H inbred strain^{4,6} and thus in various combinations with the G strain indicated in Tables 1–3 it was possible to use the T6 chromosome marker because CBA/H and CBA T6T6 inbred strains are immunologically uniform. Because of closed colony conditions the G strain has greatly reduced heterozygosity⁴ also allowing intra-strain transplantation.

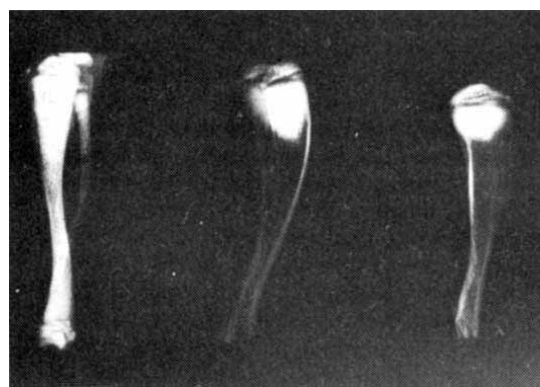


Fig. 1 Radiographs of osteopetrotic tibias ($\times 8$). Left, 28-d-old untreated G *mi* mouse. Note absence of medullary cavity. Centre, G *mi* mouse injected intraperitoneally at birth with haematopoietic cells. At 175 d partial resorption ($\pm$) had occurred but the metaphysis and submetaphyseal shaft were osteopetrotic. Right, G *mi* mouse injected intravenously at 19 d with haematopoietic cells and killed 29 d later. Resorption was subtotal (+), only the metaphysis was osteopetrotic.

In the three experiments reported here the donated hybrid cells (T6T6 $\times$ G)_{F1} were unable to react immunologically against the host—except by virtue of the H-Y antigen to which the stocks are poor responders. On the other hand, in some instances the host could formally react against non-H-2 antigens of the donated cells.

Resorption in shaft bones was evaluated at the times shown in Tables 1–3, and estimated arbitrarily by radiology and histology as 'partial' ($\pm$) when resorption had clearly started but which was far from complete, and 'sub-total' (+) when it was approaching completion. In our hands, a patch of osteopetrosis usually persisted at the growth plate of long bones (Fig. 1). The cytogenetic status was also estimated at the times shown in Tables 1–3. The behaviour of vertebrae, ribs and membrane bones are not described here. Serious defects such as blindness and failure of eruption of teeth were unaffected.

The results of the three experiments were as follows: (1) Microphtalmic G mice injected intraperitoneally at birth with bone marrow from normal hybrid (T6T6 $\times$ G)_{F1} mice showed subtotal or partial resolution of the osteopetrosis within 2 months. Only low proportions of T6-marked donor cells were detected in the spleen and bone marrow (Table 1). Persistence of donor cells, formally histoincompatible, may be attributed to immunological tolerance of the newborn to non-H-2 antigens. (2) In older mice injected intravenously, the resolution of the osteopetrosis was partial and in none were donated cells detected a month and more after treatment (Table 2).

Table 1 Chimaerism and results of treatment of newborn microphthalmic mice by intraperitoneal injection of allogeneic bone marrow suspension from phenotypically normal T6-marked mice

<i>mi</i> no.	Host stock	Host age and sex	Donor stock and sex	Cell dose ($\times 10^{-6}$)	% Dividing donor stock cells in host		Degree of resolution of osteopetrosis at time (d)
80	G	< 24 h ♀	(T6T6 $\times$ G) F_1 ♀	5	Spleen	1	+ at 55
					Bone marrow	0	
81	G	< 24 h ♂	(T6T6 $\times$ G) F_1 ♀	5	Spleen	2	+ at 56
					Bone marrow	1.5	
82	G	1 d ♂	(T6T6 $\times$ G) F_1 ♀	7	Spleen	2	$\pm$ at 58
					Bone marrow	0	
83	G	1 d ♀	(T6T6 $\times$ G) F_1 ♀	7	Spleen	2	+ at 58
					Bone marrow	1	

Microphthalmic mice are difficult to maintain; as they are edentulous, they are weaned onto a wet mash. The T6-marked cells were detected by a standard cold spread method⁷ and 100–300 metaphase plates were scored in all preparations. The T6 marker detects dividing cells but does not reveal their identity.

Table 2 Results of treatment of weanling microphthalmic G mice by intravenous injection of allogeneic bone marrow suspension from phenotypically normal T6-marked mice

<i>mi</i> no.	Host stock	Host age and sex (d)	Donor stock and sex	Cell dose ($\times 10^{-6}$)	% Dividing donor stock cells in host		Degree of resolution of osteopetrosis at time (d)
181	G	♀ 18	(T6T6 $\times$ G) F_1	5	Spleen	0	— at 35
					Bone marrow	—	
182	G	♀ 18	(T6T6 $\times$ G) F_1	5	Spleen	0	$\pm$ at 62
					Bone marrow	—	
183	G	♂ 18	(T6T6 $\times$ G) F_1	5	Spleen	0	+ at 90
					Bone marrow	0	
184	G	♀ 22	(T6T6 $\times$ G) F_1	47	Spleen	—	$\pm$ at 117
					Bone marrow	0	
185	G	♂ 22	(T6T6 $\times$ G) F_1	47	Spleen	0	$\pm$ at 117
					Bone marrow	0	
189	G	♀ 21	(T6T6 $\times$ G) F_1	47	Spleen	0	$\pm$ at 46
					Bone marrow	0	
190	G	♀ 20	(T6T6 $\times$ G) F_1	47	Spleen	0	$\pm$ at 46
					Bone marrow	0	
191	G	♂ 19	(T6T6 $\times$ G) F_1	47	Spleen	0	$\pm$ at 29
					Bone marrow	0	

Table 3 Chimaerism and resolution by parabiosis of mature microphthalmic mice with normal (T6T6 $\times$ G) F_1 hybrids

<i>mi</i> no.	Osteopetrotic stock and sex		Normal stock partner and sex	Days in parabiosis	% T6-marked dividing cells in petrotic mice		Degree of resolution of osteopetrosis at time (d)
35	G	♂	(T6T6 $\times$ G) F_1 ♂	14	Spleen	no result	$\pm$ at 120
					Bone marrow	0	
153	(G $\times$ CBA) F_1 ♂		(T6T6 $\times$ G) F_1 ♂	13	Spleen	32	$\pm$ at 120
					Bone marrow	3	
155	(G $\times$ CBA) F_1 ♂		(T6T6 $\times$ G) F_1 ♂	13	Spleen	8	+ at 120
					Bone marrow	1	
					Thymus	3	
					Blood	32	
156	(G $\times$ CBA) F_1 ♂		(T6T6 $\times$ G) F_1 ♂	13	Spleen	15	$\pm$ at 120
					Bone marrow	0	
					Thymus	5	
					Blood	29	

Mice were anaesthetised with avertin and parabiosed by the coelomic method. Braided silk (5/0) was used to suture the abdominal walls and the skin incision was closed with metal clips. At separation the normal anatomy was restored. Pairs were housed individually and received food pellets, wet mash and tap water *ad libitum*. To increase the number of metaphases for scoring, this group received sheep red blood corpuscles intraperitoneally 4 d before being killed. Previous experiments had shown that while the bone marrow was mitotically active the spleens were not. Separated leukocytes from the peripheral blood were cultured by Festenstein's method⁸.

(3) Four microphthalmic mice, after parabiosis to normal (T6T6×G)F₁ hybrids, showed partial resolution of osteopetrosis. Four months later, cells were detected in the haematopoietic tissues in the three syngeneic matchings, the greater proportion in the spleens and peripheral blood no doubt being due to antigenic stimulation (Table 3).

The results indicate that in a syngeneic combination (3) or when immunological tolerance of the newborn can be invoked in allogeneic matches (1), donated cells may persist in the microphthalmic recipient but in very small proportions. In the allogeneic match (2) no such cells were found 1 month later (compare the loss of grafts after 1 month in similar skin grafting experiments⁴). Nevertheless all groups showed resolution of the osteopetrosis to a greater or lesser degree in individuals.

It is possible that the weanlings in group (2) and the one successful case of parabiosis across an immunological barrier, where no donated cells were subsequently found, did have small and undetected populations of donor cells, which further experiments with mitotic stimulants may reveal. For the present, however, we postulate that allogeneic donor cells were eliminated but during their limited period of residence had caused considerable resorption of bone by providing osteoclastic precursors or had triggered the recipient's osteoclasts into effective function, comparable to the T-B cell-cell interactions of immunology. Experiments are in progress to investigate whether the resolution of osteopetrosis is permanent in these conditions, which would support the 'trigger' theory.

In experiment (1) and even in (3) where the spleen and separated leukocytes from the peripheral blood were under stimulation by foreign antigen, the donor population was in a minority and there was no 'take over', as would be presumed to have occurred in Walker's⁵ experiments where the recipients had been lethally irradiated before treatment.

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Cellular localisation of human urogastrone/epidermal growth factor

HUMAN urogastrone (hUG) is a polypeptide prepared from urine and is chemically similar to mouse epidermal growth factor (mEGF) prepared from submandibular salivary glands. Both these substances stimulate fibroblast proliferation in tissue culture systems. hUG and mEGF inhibit gastric acid secretion in experimental animals and hUG produces an identical effect in man. We show here, using immunofluorescent techniques,

that hUG is present in the duct cells of human submandibular gland and in Brunner's gland cells in the first part of the duodenum. This work suggests that these polypeptides may have a role in mucosal growth and control of gastrointestinal secretion.

The fact that peptic ulcer undergoes remission in pregnancy¹ led to the discovery of a powerful inhibitor of gastric acid secretion termed urogastrone in urine. This inhibitor has been shown to consist of a single polypeptide chain of 53 amino acids containing three disulphide bonds². Independently, Cohen and his colleagues³ have isolated a substance from mouse submaxillary glands known as epidermal growth factor (mEGF),

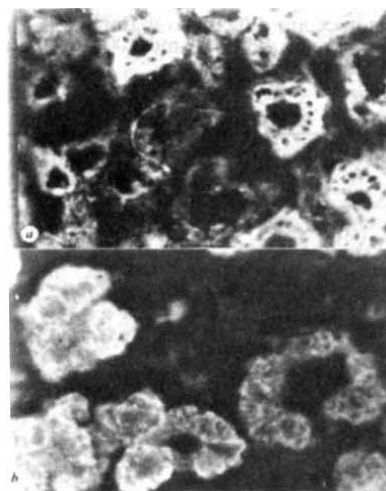


Fig. 1 Fluorescence can be seen as the light areas in *a*, the cells of the ducts of the submandibular glands and *b*, in the cells of the glands of Brunner in the first part of the duodenum.

which is closely related structurally to urogastrone and in biological activity it seems to be identical⁴. Both are powerful mitogens causing epithelial proliferation and keratinisation of squamous epithelial cells. They both inhibit gastric acid secretion and furthermore, they share common receptors in cultured human fibroblasts⁵. Preliminary physical data on a human epidermal growth factor (hEGF)⁶ suggests that the material in question is urogastrone².

Purified human urogastrone is an effective inhibitor of gastric acid secretion in normal male subjects⁷ and in patients with duodenal ulcer⁸ and Zollinger-Ellison syndrome, a disease due to an abnormally high circulating level of the hormone gastrin⁹. The cells of origin, storage or secretion of urogastrone, however, have not been identified in man. Urogastrone can be detected in human urine after a variety of surgical ablative procedures¹⁰, whereas (mEGF) has been found in specialised cells of the submandibular glands¹¹, and this finding prompted the present enquiry.

Antibodies to hUG were raised in rabbits¹². Specimens of the following normal human tissues were placed in polythene bags immediately after surgical excision, fixed in liquid nitrogen at -70 °C for 10 min, and were subsequently stored at -25 °C for further processing: submandibular salivary glands, parotid salivary glands, oesophagus, antrum and fundus of stomach, duodenum, jejunum, colon, breast, pancreas, ovary, adrenal, thyroid, kidney, bladder, liver, prostate, smooth muscle, skeletal muscle, cardiac muscle and thymus were studied. Frozen sections were cut and the slides exposed for 20 min to specific rabbit hUG antiserum without dilution and at 1 : 20 dilution using saline. The slides were washed for 20 min, and sheep anti-rabbit fluorescein-labelled immunoglobulin (Wellcome) was applied for a further 20 min followed by a final rinse. The

sections were then examined under ultraviolet light. Control sections from the same tissues were processed using nonspecific rabbit serum and also in a separate series using hUG absorbed specific rabbit antiserum. Adjacent sections of all tissues were stained with conventional haematoxylin and eosin to allow identification of cell types more easily in comparison with the fluorescein-labelled sections. An alternative method of fixation using buffered picric acid formaldehyde (pH 7.3) for 48 h before section cutting and application of the double layer fluorescence technique gave similar results to those obtained with the cryostat material. Fluorescence was seen in the cells of the ducts of human submandibular salivary glands and in the cells of Brunner's glands of the duodenum (Fig. 1a, b). Examination of sections from all the other tissues and of all control sections failed to reveal any appreciable fluorescence. An alternative freeze-drying and formaldehyde vapour fixation technique followed by immunostaining procedures has been reported¹³ to give an extremely clear picture of localisation of mEGF, but is much more time consuming than the methods outlined here for human tissues.

Our tests indicate that immunoreactive urogastrone/epidermal growth factor is present in specialised cells of the human submandibular salivary gland and also in the cells of the glands of Brunner in the duodenum. These findings may explain why in previous studies significant amounts of urogastrone could be detected in urine following different surgical ablation procedures in man¹⁰. No case examined so far has involved ablation of these two specific areas.

Our results also raise the question of the nature of the gastric acid inhibitory hormone 'enterogastrone', said to be liberated from the duodenum by fat ingestion in the studies of Kosaka and Lim¹⁴ (reviewed in detail by Gregory¹⁵). Could this 'enterogastrone' be urogastrone, a well-established potent inhibitor of gastric acid secretion and now localised in Brunner's glands? Furthermore, it is difficult to avoid speculation that the presence of a powerful growth factor at two sites in the gastrointestinal system is probably related to the known rapid turnover of the cells lining the gut¹⁶.

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Renal actions of prostacyclin

A NEW and important member of the arachidonate-thromboxane-prostaglandin system, prostacyclin (PGI₂), has recently been isolated¹⁻³. This bicyclic prostaglandin has been shown to be synthesised from the prostaglandin endoperoxides PGG₂ and PGH₂, by arteries, veins, heart and other tissues in several species, including man¹⁻⁷. PGI₂ is a powerful inhibitor of platelet aggregation and relaxes arterial strips^{1,2,4,5}. It is 30 times more potent than PGE₁ as an inhibitor of platelet aggregation, and in the intact dog promotes a dramatic reduction in systemic blood pressure^{1,2,8,9}. It has also been suggested that PGI₂ is a potent coronary vasodilator and anti-atherogenic compound¹⁰⁻¹², and that it plays an essential part in the homeostasis of the cardiovascular system^{1,2,4,5,11,12}. The kidneys receive a large portion of the cardiac output and have an extensive vascular tree, and so PGI₂ may be extremely significant in regulating kidney function. Human and rabbit renal cortical microsomes have been found to convert PGG₂ to PGI₂ (ref. 13), but nothing is known of the renal effects of PGI₂. Therefore, this study was designed to evaluate the effects and significance of an intrarenal infusion of PGI₂ on renal haemodynamics, urinary function and renal vein plasma renin activity, and to compare these effects with those produced by PGE₂ and PGD₂, two prostaglandins known to be synthesised by the kidney and to affect renal function.

Table 1 shows the effects of intrarenal infusions of PGI₂, PGD₂ and PGE₂. All three prostaglandins markedly elevated renal blood flow. The increases in blood flow produced by PGI₂ and PGD₂ were due to an enhanced blood flow to both the outer and inner cortical regions as shown in Table 1. The increases in blood flow to the inner cortex were greater than those in blood flow to the outer cortex, thus shifting the intrarenal distribution of blood flow to the inner cortex. Although no values for the PGE₂ experiments are shown, radionuclide-labelled microspheres were used in one experiment, and results similar to those seen with PGI₂ and PGD₂ were obtained.

All three prostaglandins produced similar increases in renal blood flow without a change in glomerular filtration rate. Their effects on urinary function, however, were dissimilar (Table 1), both PGI₂ and PGE₂ promoting a natriuresis and diuresis, and PGD₂ having no significant effect on the urinary flow rate and the fractional excretion of sodium. In addition, PGI₂ and PGE₂ but not PGD₂, significantly elevated the excretion rate of potassium (not shown). At no time during the infusion of these prostaglandins were changes noted in systemic blood pressure, heart rate or contralateral renal function, indicating that at the dosage employed, the effects induced by these prostaglandins were restricted to the infused kidney. In two experiments, the PGI₂ solvent and the PGI₂ metabolite, 6-keto-PGF_{1α}, were infused separately and found to have no significant effect on renal blood flow, urinary output or renal vein plasma renin activity. This implies that neither the solvent nor the metabolite were responsible for the renal effects induced by PGI₂.

In a previous report, we demonstrated that the infusions of PGD₂ and PGE₂ at a 10-fold higher rate of 0.4 µg per kg of body weight per min produced changes in renal function qualitatively similar to the effects elicited by PGD₂ and PGE₂ in this study¹⁴. No systemic effects were noted. PGI₂ at this higher rate of infusion (0.4 µg per kg per min) decreased systemic blood pressure and increased heart rate. This indicates that PGI₂ leaving the kidney was not effectively metabolised by the lungs, and is consistent with the observation made earlier by Waldman *et al.*¹⁵ that the vasodepressor response of PGI₂ in the dog is not diminished by passage through the lungs, in contrast to a dramatic reduction in the vasodepressor effect of PGE₂. This phenomenon might be explained in one of three ways. First, PGI₂ undergoes little metabolism in the lung;

second, PGI_2 undergoes some metabolism in the lung, but because of its marked potency, enough escapes to cause systemic effects; third, the amount of PGI_2 reaching the lung during the higher infusion rate exceeded the capacity of the lung to metabolise it. These observations suggest that if PGI_2 is formed and released by the kidney in sufficient quantity, it might function as a circulating vasodepressor substance.

The pattern of blood flow changes induced by the three prostaglandins is similar to that produced by the prostaglandin precursor, arachidonic acid, and other renal vasodilators^{16,17}. Their similar haemodynamic effects, but differing effects on urinary function suggest that PGI_2 and PGE_2 , but not PGD_2 , either directly and/or indirectly inhibit sodium and water reabsorption. Although the present experiments do not elucidate the mechanism by which PGI_2 promotes a natriuresis and diuresis, PGE_2 has been shown to inhibit directly the reabsorption of sodium in the nephron^{18,19}.

Of the three prostaglandins, only PGD_2 significantly elevated renal vein plasma renin activity when infused at a rate of $0.04 \mu\text{g per kg per min}$. Vander²⁰ also observed that PGE_2 infusion in a similar dose did not alter renin release. In a previous series of experiments, however, we found that when PGE_2 was infused at a 10-fold higher rate ($0.4 \mu\text{g per kg per min}$) renal vein plasma renin activity was increased¹⁴; Yun *et al.*²¹ also found this. A comparison of the haemodynamic and urinary effects of PGE_2 at the two doses (0.04 and $0.4 \mu\text{g per kg per min}$) used in our studies revealed that the higher dose produced an increase in renal blood flow which was twice as great as that produced by the lower dose, while the diuretic and natriuretic effects were not appreciably different at the two dose levels. Previous demonstrations of increased renin production following administration of arachidonic acid or PGE_2 led to the suggestion that prostaglandins may act directly on the juxtaglomerular cells to release renin. This conclusion

has been questioned, as it was pointed out that the accompanying natriuresis *per se* may have triggered renin release by presenting more sodium to the macula densa²². The present observation might be explained by precisely the opposite argument, that the renal haemodynamic effects induced by PGI_2 and PGE_2 are promoting renin release whereas the natriuresis is inhibiting release. Our additional experiments have used a non-filtering kidney model in which the delivery of sodium to the macula densa is prevented; the infusion of either PGI_2 or PGE_2 at the lower dose ($0.04 \mu\text{g per kg per min}$) then increased renin release. In the intact kidney, either an elevated sodium load at the macula densa as suggested by Vander²³, or an action of PGE_2 and PGI_2 to reduce ion flux across the macula densa as postulated by Thureau *et al.*²⁴ may have blunted an increase of renin release. The stimulation of renin release induced by PGD_2 is unopposed because of the absence of a natriuresis.

Our evaluation of PGI_2 indicates that when infused intrarenally it produces changes in renal haemodynamics and urinary function which are qualitatively identical to those produced by PGE_2 . It is impossible at present to state which prostaglandin (PGI_2 , PGE_2 or PGD_2) is the major renal prostaglandin mediating the effects of endogenous arachidonate released following neural and hormonal stimulation. This will be resolved by the future development of analytical techniques which will permit measurement of the levels of renal prostaglandins in physiological conditions. However, it is noteworthy that PGI_2 escapes metabolism by the lung; if the kidney is a major source of PGI_2 it may have significant effects beyond that organ. Conversely, if arterial levels of PGI_2 are sufficiently high, production of PGI_2 elsewhere in the body may have significant effects on renal function. It should be noted, however, that PGI_2 did not exhibit any superior potency to PGE_2 in its effects on renal function. Finally, we obtained

Table 1 The effects of intrarenal infusions of PGI_2 , PGD_2 and PGE_2

Infusion periods	RBF (ml per min per g of kidney)	OCBF (ml per min per g of tissue)	ICBF (ml per min per g of tissue)	RF	$\dot{V}$ ($\mu\text{l per min per g}$ of kidney)	FE_{Na}^+ (%)	RVPRA ng per ml per h
	N = 9	N = 8	N = 8	N = 8	N = 9	N = 9	N = 5
Control	4.1 ± 0.6	8.0 ± 1.2	4.4 ± 0.6	1.9 ± 0.1	7 ± 1	0.94 ± 0.25	5.6 ± 1.5
PGI_2	$6.1 \pm 0.7^*$	$10.7 \pm 0.8^*$	$9.7 \pm 0.8^*$	$1.2 \pm 0.1^*$	$15 \pm 2^*$	$2.03 \pm 0.56^*$	7.0 ± 2.2
	N = 8	N = 6	N = 6	N = 6	N = 8	N = 8	N = 6
Control	4.2 ± 0.6	7.9 ± 1.1	4.4 ± 0.8	2.0 ± 0.2	20 ± 4	1.40 ± 0.46	3.8 ± 1.3
PGD_2	$7.3 \pm 1.2^*$	$14.9 \pm 2.7^*$	$13.3 \pm 2.7^*$	$1.2 \pm 0.1^*$	25 ± 4	1.75 ± 0.50	$8.8 \pm 2.2^*$
	N = 7				N = 7	N = 7	N = 6
Control	4.4 ± 0.5	—	—	—	12 ± 3	1.58 ± 0.52	3.9 ± 1.5
PGE_2	$6.2 \pm 0.7^*$	—	—	—	$38 \pm 10^*$	$3.54 \pm 0.88^*$	4.6 ± 1.6

Values (right renal) are expressed as the mean $\pm$ s.e.m.; N, no. of animals; *, $P < 0.05$ as determined by the paired *t* test. RBF, renal blood flow; OCBF, outer cortical blood flow; ICBF, inner cortical blood flow; RF, ratio of cortical blood flows (outer cortical to inner cortical blood flows); $\dot{V}$, urinary flow rate; FE_{Na}^+ , fractional excretion of sodium; RVPRA, renal vein plasma renin activity. OCBF, ICBF, RF and RVPRA were not determined in all experiments because of technical difficulties. OCBF, ICBF and RF mean values are not shown for PGE_2 experiments, because intrarenal blood flow was not determined. Mongrel dogs of either sex ($N = 22$) were fasted 18 h before the experiment and allowed free access to tap water. The animals were anaesthetised with intravenous pentobarbital (30 mg per kg of body weight) and placed on a positive pressure respirator (Harvard Apparatus). Indwelling polyethylene catheters were inserted into the following vessels; the right femoral artery for monitoring of systemic blood pressure and collection of blood samples, the left femoral artery for advancement into the left ventricle, the left femoral vein for advancement into the right renal vein and the right femoral vein for the infusion of inulin to measure the glomerular filtration rate. A suprapubic midline abdominal incision was made and both ureters were cannulated. A right retroperitoneal flank incision was performed, and a hydraulic cuff (*In Vivo* Metric Systems) and electromagnetic flowprobe (Biotronix Laboratories) were placed on the renal artery. A 25-gauge infusion needle was inserted into the artery proximal to the flowprobe and hydraulic cuff, and normal saline was infused at 0.5 ml min^{-1} . One hour was allowed for stabilisation of renal function. Each experiment comprised two 15-min infusion periods (control saline infusion followed by PGI_2 , PGD_2 or PGE_2 ($0.04 \mu\text{g kg}^{-1} \text{ min}^{-1}$) infusion), with three 5-min urine collections in each period. After the start of the second urine collection, arterial samples for inulin, sodium and potassium and renal vein samples for renin were collected. Midway through each period radionuclide-labelled microspheres ($15 \pm 5 \mu\text{m}$ ^{85}Sr , ^{141}Ce and ^{51}Cr , New England Nuclear) were introduced into the left ventricle with the exception of the PGE_2 experiments. PGD_2 and PGE_2 were dissolved in ethanol and stored at -20°C until the day of the experiment, when the ethanol was evaporated off and the prostaglandins were dissolved in normal saline. PGI_2 was chemically synthesised according to the method of Corey *et al.*²⁵. It was dissolved in water and stored at -70°C until several hours before the experiment; an aliquot of the stock solution was then dissolved in a Tris buffer at pH 9. At the end of this prostaglandin infusion period, the animals were killed and the kidneys removed, weighed and sectioned according to the method of Slotkoff *et al.*²⁶ for counting of radioactivity. Renin levels were measured by radioimmunoassay.

data which suggests that the net effect of either PGI_2 or PGE_2 on renin release is the summation of its stimulatory action by means of haemodynamic changes and/or a direct stimulation of the juxtaglomerular apparatus, versus a dampening effect produced by altered sodium transport at the macula densa.

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Temperature-induced phase shift of daily rhythm of serum prolactin in gulf killifish

DAILY variations in circulating levels of the pituitary hormone prolactin have been reported in several vertebrates, including fishes^{1–4}. In some animals, the 24-h rhythm changes seasonally with respect to the time of day that maximum and minimum prolactin levels occur^{4,5}. It has been hypothesised that this seasonal change in phase of prolactin rhythm is an important component of the mechanism controlling seasonality in vertebrates⁶. Because water temperature is generally considered the principal environmental regulator of seasonal changes in reproduction and metabolism in many fishes, including the gulf killifish *Fundulus grandis*⁷, we determined the daily rhythm of serum prolactin concentrations in fish held at temperatures that are stimulatory (20 °C) or inhibitory (28 °C) for reproductive development. We found that an increase in water temperature from 20° to 28 °C phase shifts the daily variation of serum prolactin with respect to the daily photoperiod in *F. grandis*.

There were variations in serum prolactin concentration in gulf killifish maintained at water temperatures of either 20° or 28 °C ($P < 0.01$; $P < 0.05$, one-way analysis of variance). However, the phase relationships of the peaks and troughs of the two rhythms differed with respect to the daily photoperiod 12 h light and 12 h dark (12L:12D). Thus, the peak

level of prolactin in fish acclimated to 20 °C occurred at 1400 h (8 h after the onset of light) whereas the prolactin peak in fish acclimated to 28 °C occurred at 0600 h (onset of light) (Fig. 1). There was a highly significant interaction between the temperature of acclimation and the time of day of sampling ($P < 0.01$,

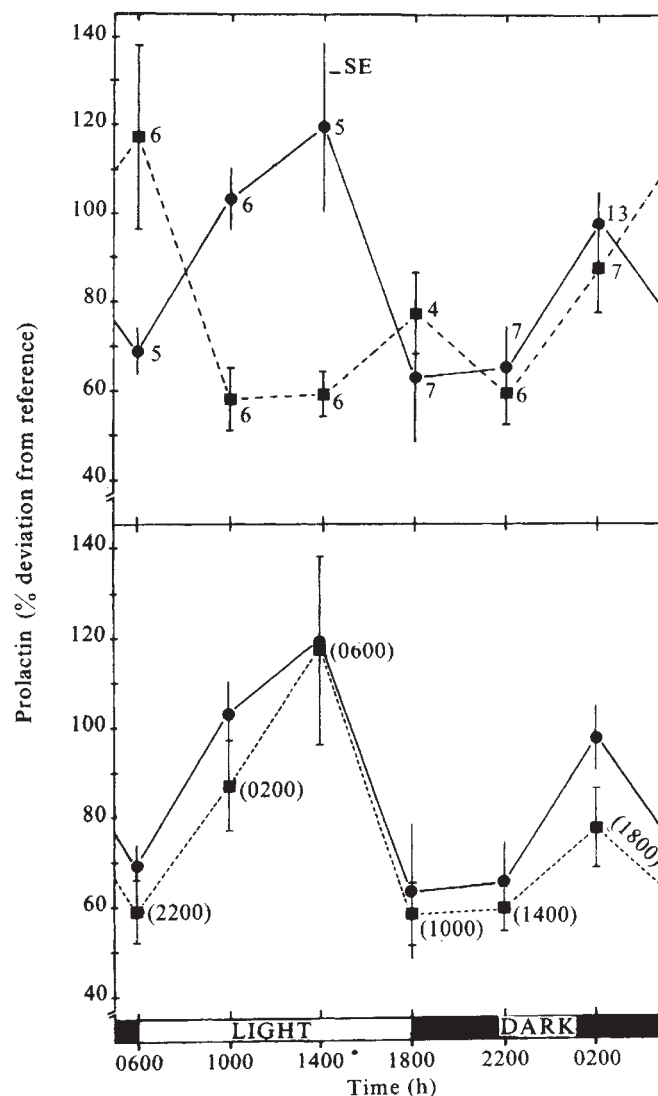


Fig. 1 Serum prolactin levels of gulf killifish acclimated to either 20 °C (●) or 28 °C (■). Both groups of fish were kept in a 12L:12D photoperiod regime—with light onset at 0600 h. The upper graph depicts the two unadjusted rhythms; sample size is adjacent to the mean prolactin levels, vertical bars represent standard error of the mean. In the lower graph the points depicting the prolactin rhythm of the 28 °C acclimated fish have been adjusted by 8 h to demonstrate the similarity in form of the two rhythms. Actual sampling times of the 28 °C acclimated fish are shown in parentheses. Fish of both sexes were collected from Grand Isle, Louisiana in February and maintained indoors in 70-l aquaria which received filtered and aerated water (salinity 3‰) and approximately 2 g of flake food (TetraMin) once daily. Routine maintenance and feeding were done at random times during the day. After a 2-week preliminary acclimation at 20 °C and 12L:12D (approximately the March photoperiod regime of Grand Isle) fish were divided into two groups which received either 20° or 28 °C, 12L:12D and 3‰ salinity were maintained for both groups. After an additional 2 weeks of acclimation, the fish were killed at one of six different times of day. Blood was taken by heart puncture, allowed to clot under refrigeration and centrifuged. The serum was assayed with a double antibody technique utilising an antiserum to pollock prolactin and iodinated ovine prolactin¹². Because killifish prolactin was not available, we were unable to determine absolute levels of prolactin. However, a pooled reference standard was run which allowed us to ascertain relative differences in circulating titres of prolactin. Mean levels of prolactin were not significantly different between the sexes.

two-way analysis of variance). This interaction is due to differences in the phase relationship of the two rhythms. The significant interaction can be eliminated by shifting the rhythm of the fish acclimated to 28 °C to 8 h later in the day (Fig. 1). The mean serum prolactin concentrations (mean of all values throughout the day) did not differ between the two groups.

Our work reconfirms the often cited warning to researchers of the problems inherent in sampling at one time of day with the added caution that environmental factors other than photoperiod (for example, temperature) may influence hormone rhythms considerably. Further, the phase shift in the prolactin rhythm we induced by a temperature change may have important implications in the understanding of the seasonal physiology of the gulf killifish. Increased water temperature causes gonadal regression in several species of fish⁸ including *F. grandis*. Gulf killifish collected from their natural environment have ripe gonads when ambient temperatures are 20 °C (early March) and regressing gonads when ambient temperatures reach 28 °C (May–June) and higher (July–August)^{7,9,10}. In our experiment male killifish acclimated to 28 °C had a significantly lower gonadosomatic index ((gonad weight/body weight) × 100) than those acclimated to 20 °C ($P < 0.05$ Student's *t* test). Apparently, the effects of temperature on the gonads may be mediated by altering the phase of the prolactin rhythm. In male killifish maintained on a 15L:9D regimen, daily injections of prolactin 8 h after the onset of light (the time of peak prolactin levels in the fish acclimated to 20 °C) stimulated the reproductive system whereas prolactin injections at other times of day including light onset (the time of peak prolactin in fish acclimated to 28 °C) were ineffective⁷.

The annual cycle of reproduction in teleost fishes is regulated by changes in daylength, temperature or a combination of both⁸. The strategy used seems to depend more on ecological demands than on phylogenetic relatedness. As in birds and mammals⁸, circadian mechanisms control reproduction in a photosensitive teleost¹¹. Our results indicate that temperature control of reproduction in an ectothermic thermosensitive species may also be mediated in part by circadian systems.

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Cyclic AMP and noradrenaline sensitivity fluctuations in regenerating newt tissue

Limb regeneration in the adult newt is dependent on the nervous system^{1,2} but the biochemical events involved are not well understood. A role for catecholamines (CA) seems likely since histofluorescence has shown that CA are more abundant in regenerates than in other newt limb tissues³. It has also been shown that reserpine, which promotes the release of CA from nerve terminals, accelerates regeneration⁴, whereas chlorpromazine⁵, guanethidine⁵ (both CA release blockers) and α -methyl-*p*-tyrosine⁶ (CA biosynthesis inhibitor) inhibit it. Studies suggest that in many biological systems the CA effects are linked to the activation of membrane adenylate cyclase with subsequent production of cyclic AMP⁷. It has also been suggested that nerves may transmit trophic information by the stimulation of this enzyme^{8–11}. To test this hypothesis further, regenerates were isolated either for direct measurements of cyclic AMP or for stimulation with CA *in vitro*¹². This method avoids the possible nonspecific effects due to systemic administration of CA. We now report that the fluctuations of cyclic AMP found at the various stages of regeneration correlate with noradrenaline (NA) sensitivity changes.

Adult newts *Triturus cristatus* were housed and handled and their limbs amputated as before⁶. Characterisation of the stages was performed on a morphological basis¹³, which is the most reliable criterion for specimen selection¹⁴. In order to measure true endogenous levels of cyclic AMP, isolated regenerates were collected in 0.25 ml of batracian Ringer¹⁵ and immediately heated in a boiling water bath for 10 min. A pool of 3–10 regenerates, depending upon the amount of regenerating tissue, was routinely used. The cyclic AMP accumulation induced by noradrenaline (or

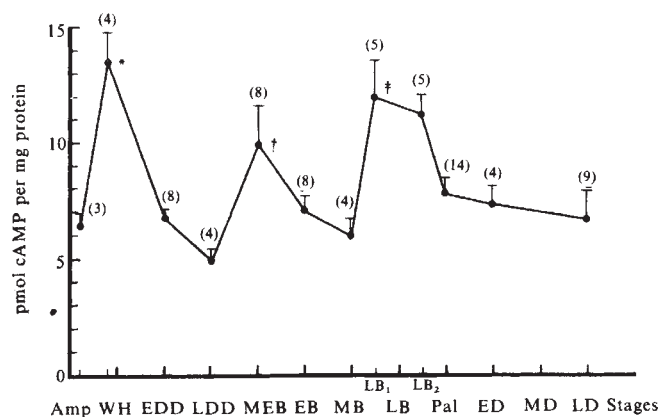


Fig. 1 Endogenous cyclic AMP levels (pmol per mg protein) in newt forelimb regenerates at various stages of development. The various stages were designed as described elsewhere¹³. Amp = amputation, WH = wound healing, EDD = early dedifferentiation, LDD = late dedifferentiation, MEB = moderate early bud, EB = early bud, MB = medium bud, LB₁ = early LB, LB₂ = late bud, LB₂ = late LB, PAL = palette, ED = early digits, MD = medium digits, LD = late digits. Regenerating tissue was removed with iridectomy scissors from live animals placed under binocular magnifying glass. After removal, the tissue was immediately plunged into a glass homogeniser (containing 0.25 ml of Ringer solution) kept in boiling water. The heat inactivation was continued for 10 min. A pool of 10 (WH) to 3 (LD) regenerates was used for 1 homogeniser, the total number of experimental tubes at various stages being given in parentheses (= *N*). The tissues were then homogenised, at 4 °C, and centrifugation (4,000 r.p.m.) was performed at the same temperature. The pellets were used for protein determination²². Duplicate samples (50 µl) of the supernatant were taken for the measurement of cyclic AMP using a saturation assay method²³. Values are mean ± s.e.m. Statistical analysis was performed using Student *t* test for unpaired data. *, Significantly different from previous ($P < 0.01$) and next ($P < 0.001$) stages. †, Significantly different from LDD ($P < 0.05$). ‡, Significantly different from MB and Pal stages ($P < 0.05$).

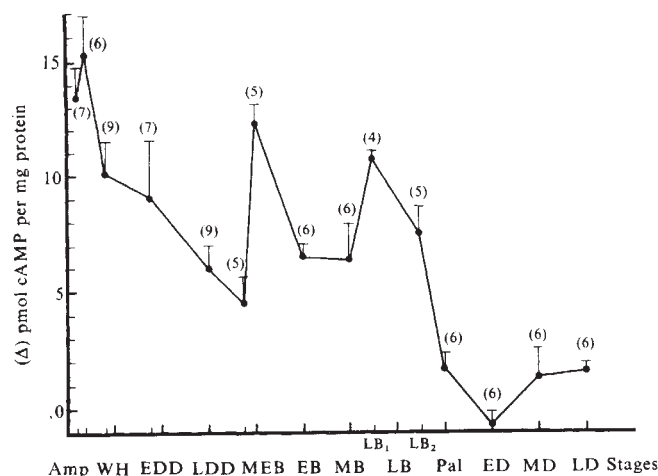


Fig. 2 Cyclic AMP increase (pmol per mg protein) induced by 10^{-4} M noradrenaline in newt forelimb regenerates at various stages of development. Pools of isolated regenerates were pre-incubated for 20 min at 35°C in 0.25 ml of Ringer solution¹⁵. After addition of theophylline (final concentration 5×10^{-3} M), which prevents the hydrolysis of cyclic AMP by phosphodiesterase, an incubation was carried out at 35°C for 10 min (total volume 0.33 ml) with noradrenaline. Controls were incubated in the absence of noradrenaline. The tissues were then inactivated by heat (see Fig. 1 for other experimental details). The increase in cyclic AMP above control is expressed as Δ . Values are mean (of Δ) + s.e.m. (vertical bars) for the number of samples shown in parentheses ($= N$). In the absence of noradrenaline, the cyclic AMP values (pmol per mg protein) were 1.9 (WH 24 h), 6.8 (WH 48 h), 1.9 (WH 4 d), 3.4 (WH-EDD), 2.6 (LDD), 3.7 (late LDD), 2.7 (MEB), 3.3 (EB), 3.7 (MB), 4.0 (LB), 3.2 (LB_2), 4.2 (Pal), 4.4 (ED), 4.0 (MD) and 3.6 (LD). The abbreviations of the various stages are given in Fig. 1.

other adrenergic agonists) was measured as before¹². Some experiments were performed in the presence of an α - or β -adrenergic antagonist. Figure 1 shows that endogenous cyclic AMP levels fluctuate as a function of the stage of regeneration. Three distinct peaks of cyclic AMP were found and these occurred at the wound-healing (WH), at the moderate early bud (MEB) and at the late bud (LB) stages. The lowest values of cyclic AMP were found at the late dedifferentiation stage (LDD). Three peaks of cyclic AMP accumulation were also observed when isolated regenerates were exposed to 10^{-4} M NA (Fig. 2). In some experiments, pieces of tissue located under the wound were also exposed to NA and the results compared to those obtained with regenerates of the same forelimbs (Fig. 3). Regenerating tissues reacted to NA by a large increase in cyclic AMP concentration, while stump tissues did not. This implies that NA sensitivity differences may

exist between growing and resting limb tissues. Strikingly, the peaks resulting from NA stimulation (Fig. 2) roughly correspond to the peaks of endogenous cyclic AMP levels (Fig. 1). Incubation of regenerates at the WH and MEB stages was also performed in the presence of isoproterenol (ISO) or dopamine (DA). The former catecholamine stimulated cyclic AMP accumulation, while DA had no effect (data not shown). Moreover, in the presence of the β -adrenergic receptor antagonist, propranolol, or phentolamine, only the former was able to inhibit the cyclic AMP increases induced by NA (Table 1) or ISO (not shown). These results suggest that trophic information mediated by CA involves β -adrenergic receptors.

Thus, during the process of regeneration, fluctuation occurs in endogenous cyclic AMP levels and sensitivity of the tissue receptors to NA. It is tempting to correlate nerve activity, cell sensitivity to NA, endogenous cyclic AMP and the passage of one stage of regeneration to another. The first peak of cyclic AMP could be associated with the wound healing process (possibly linked to cell movements¹⁶) and the drop noted at LDD could be necessary for the occurrence of cell dedifferentiation. The following peaks might then be related to bursts of proliferation¹⁷, possibly linked to one or several phases of the cell cycle and to differentiation^{18,19}. Very recently, it was found that cyclic GMP levels also change during newt limb regeneration²⁰

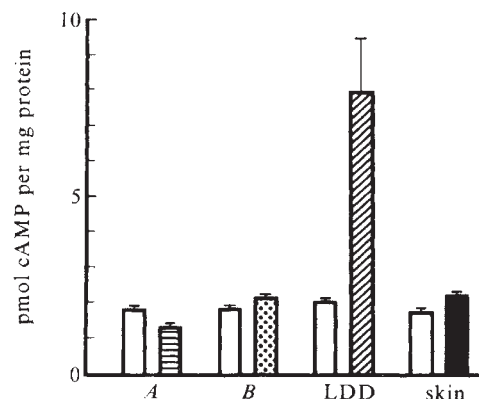


Fig. 3 The effects of 10^{-4} M noradrenaline on the cyclic AMP concentration (pmol per mg protein) in regenerating or non-regenerating tissues of the newt. Regenerates at the LDD stage were removed (see Fig. 1), as well as skin of nonregenerating tissues dissected from the region distant from the wound A, or adjacent to it B. Empty bars are controls, filled bars represent noradrenaline-treated tissues. The experimental details were given in Fig. 2. Values are mean + s.e.m.

and the low values of cyclic AMP observed at LDD (Fig. 1) seem to coincide with high levels of cyclic GMP at the same stage²⁰. Fluctuations of both cyclic nucleotides in a related but opposite way may then be needed during the process of regeneration, at least at dedifferentiation stages, and it is possible that the cyclic GMP effects are mediated by another nerve trophic factor of peptide nature²¹. Such speculations are currently under investigation using newt forelimb regenerates.

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Table 1 The effects of adrenergic receptor antagonists on the cyclic AMP accumulation induced by 10^{-4} M noradrenaline in newt regenerates of wound healing stages

WH-stage regenerates	pmol per mg protein
Control	6.0 ± 1.2 (4)
Noradrenaline	17.9 ± 2.8 (5)
Noradrenaline + phentolamine	15.8 ± 2.0 (3)*
Noradrenaline + propranolol	3.4 ± 0.3 (5)†

Control regenerates or regenerates exposed to noradrenaline were treated as described in Fig. 2. In experiments with an antagonist (final concentration 10^{-4} M), phentolamine or propranolol were added just before noradrenaline for the final 10 min incubation. Values are means $\pm$ s.e.m. for the number of samples given in parentheses ($= N$).

*Not significantly different from noradrenaline-treated regenerates ($P > 0.6$, Student's t test).

†Not significantly different from control-regenerates ($P > 0.05$) and significantly different from noradrenaline-treated regenerates ($P < 0.001$).

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Increases in cyclic GMP levels may not mediate relaxant effects of sodium nitroprusside, verapamil and hydralazine in rat vas deferens

It has been suggested that increases in cyclic GMP levels may be responsible for the promotion of contractions in a variety of smooth muscles¹⁻⁶. This suggestion was based on observations that agents known to be capable of contracting smooth muscles produced marked increases in cyclic GMP levels in the respective muscles¹⁻⁸. However, recent reports have concluded that, although increases in cyclic GMP levels do occur during contractions in some types of smooth muscle, the increases in cyclic GMP levels are not responsible for the initiation or promotion of the contractions⁹⁻¹². Part of the evidence for this conclusion was the observation that marked increases in cyclic GMP levels occurred during relaxation of smooth muscles by drugs such as nitroglycerol^{10,12}. Schultz *et al.*¹³ have shown that other smooth muscle relaxants including sodium nitroprusside, hydralazine, and D-600 (a methoxy derivative of verapamil) are all capable of increasing cyclic GMP levels in isolated segments of rat vas deferens. These authors also noted that the 8-bromo derivative of cyclic GMP was itself capable of relaxing the rat vas deferens in certain conditions¹³. It was concluded that, not only was cyclic GMP not a mediator of smooth muscle contraction, but it might in fact be responsible for the smooth muscle relaxant effects of the above drugs. We describe here studies performed in rat vas deferens. Our results are not consistent with the hypothesis that increases in cyclic GMP levels are responsible for drug-induced smooth muscle relaxation.

Schultz *et al.*⁸ have suggested that the increases in cyclic GMP levels which accompany smooth muscle contractions might be part of a negative feedback mechanism tending to reduce calcium influx or to increase calcium efflux, thus limiting or terminating the contractions. The suggestion that cyclic GMP may mediate the relaxant effects of some drugs is a logical extension of this hypothesis as, if the hypothesis is true, any drug which directly increases cyclic GMP levels in a smooth muscle should tend to lower intracellular ionised calcium levels and relax the muscle. However, in the experiments of Schultz *et al.*¹³ the effects of the relaxant drugs on cyclic GMP levels in the vas deferens were measured in the absence of extracellular calcium, and the effects of the drugs on muscle tension were not monitored. In our opinion, if direct correlations between cyclic nucleotide levels and drug-induced relaxation are to be made, it is essential that both parameters be measured. Therefore, the experiments described here were carried out in physiological salt solutions containing calcium, and the effects of relaxant drugs on both tension and cyclic nucleotide levels were monitored simultaneously in the same muscle segments.

In order to demonstrate the relaxant effects of drugs on the isolated vas deferens it was first necessary to contract the muscles. This was accomplished either by adding 30 μ M phenylephrine to the muscle baths or by exposing the muscles to 124 mM KCl¹⁰. The effects of these agents on tension developed by the muscles are shown in Fig. 1 together with the effects of verapamil and sodium nitroprusside on KCl- and phenylephrine-induced contractions. Verapamil at 20 μ M was capable of relaxing segments of rat vas deferens which had been previously contracted by KCl (Fig. 1 a) or by phenylephrine (data not shown). Pretreatment with 50 μ M verapamil for 30 s almost completely blocked the stimulatory effects of a subsequent dose of phenylephrine (Fig. 1 b). Similar results were obtained with hydralazine (not shown) although the onset of relaxation was slower than with verapamil and higher concentrations of hydralazine (1 mM) had to be used to obtain comparable degrees of relaxation. Sodium nitroprusside had no effect on contractions induced by phenylephrine (Fig. 1 c) or KCl (Table 1).

Cyclic nucleotide levels were determined in paired segments of rat vas deferens exposed to several combinations of stimulant and relaxant drugs. In agreement with previous reports⁸ cyclic GMP levels were markedly increased in segments of vas deferens exposed to KCl alone. For example, cyclic GMP levels were increased from 5.1 ± 1.6 pmol per g tissue in relaxed controls, to 31.9 ± 4.0 pmol per g tissue 5 min after KCl-induced depolarisation ($N=5$). The KCl-depolarised muscles could be relaxed by concentrations of hydralazine or verapamil which had no further effect on cyclic GMP levels (Table 1). These concentrations of hydralazine and verapamil were sufficient to completely relax the muscles within 6-10 min, although at 1 min after drug administration they were relaxed by only 16% and 38% respectively. Conversely, sodium nitroprusside failed to relax KCl-induced contractures of rat

Fig. 1 Representative tracings illustrating the effects of verapamil and sodium nitroprusside (SNP) on KCl- and phenylephrine-induced contractions of isolated rat vas deferens.

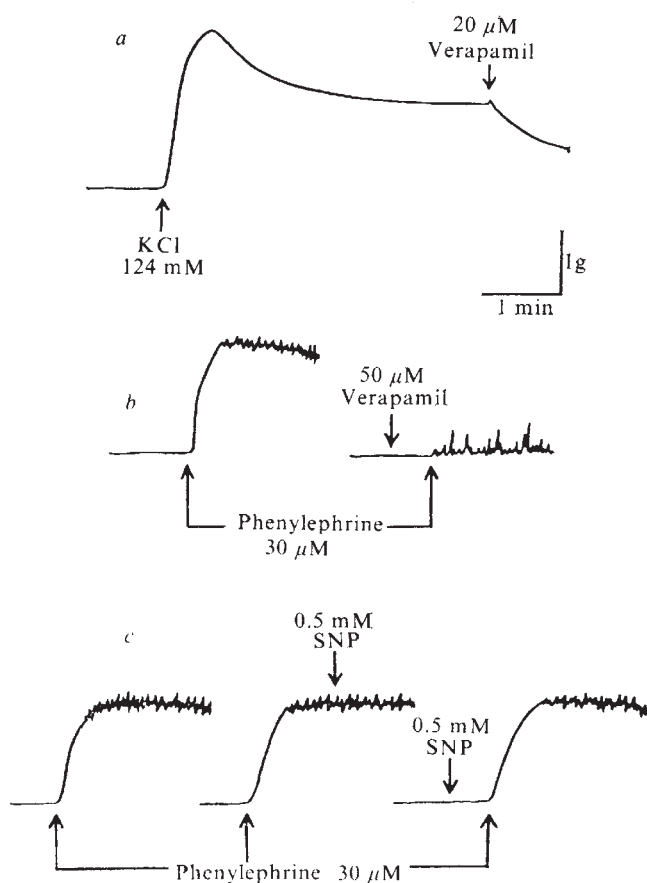


Table 1 Effects of hydralazine, verapamil and sodium nitroprusside on tension and cyclic nucleotide levels in rat vas deferens

Treatment	N	Cyclic GMP (pmol per g tissue)	Cyclic AMP (pmol per g tissue)	Relaxation (%)
KCl, 5 min (control)	5	33.0 ± 1.5	683 ± 74	—
KCl, 5 min + hydralazine last 1 min	5	33.6 ± 2.6	658 ± 31	16.4 ± 1.5*
KCl, 5 min (control)	7	31.7 ± 3.8	863 ± 88	—
KCl, 5 min + verapamil, last 1 min	7	33.1 ± 5.8	855 ± 70	37.6 ± 3.8*
KCl, 5 min (control)	6	30.2 ± 6.5	621 ± 138	—
KCl, 5 min + Na nitroprusside, last 1 min	6	67.8 ± 13.8*	646 ± 120	0.0
Phenylephrine, 2 min (control)	6	16.3 ± 3.9	717 ± 90	—
Phenylephrine, 2 min + Na nitroprusside, last 1 min	6	120.5 ± 19.6*	783 ± 89	0.0

Paired segments of rat vas deferens were equilibrated in physiological salt solution as previously described¹¹. Both muscles of a given pair were then exposed to either 124 mM KCl or 30 µM phenylephrine for 5 or 2 min respectively. One muscle from each pair was also exposed to a relaxant drug for the last minute of the experiment. Concentrations of the relaxant drugs used were: verapamil, 20 µM; hydralazine, 1 mM; sodium nitroprusside, 0.5 mM in the phenylephrine experiments and 0.1 mM in the KCl experiments. Muscles were clamp-frozen at the appropriate times and cyclic nucleotides were extracted and assayed using standard techniques¹¹. Values represent means ± s.e.m. for the number of experiments indicated (N).

*Significantly different from corresponding controls ($P < 0.001$).

vas deferens, while significantly increasing cyclic GMP levels in these preparations (Table 1). Furthermore, 0.5 mM sodium nitroprusside, which had no effect on phenylephrine-induced contractions, increased cyclic GMP levels almost eightfold over the corresponding phenylephrine controls (Table 1). No significant changes in cyclic AMP levels were seen in any of the experiments. Thus, in the case of hydralazine and verapamil we were able to markedly relax the vas deferens without changing cyclic GMP levels, and in the case of sodium nitroprusside we were able to markedly increase cyclic GMP levels without relaxing the muscles. These results are not consistent with the suggestion that the relaxant effects of these drugs are mediated by increases in tissue levels of cyclic GMP.

It might be argued that the sodium nitroprusside-induced increases in cyclic GMP levels were activating mechanisms that tend to lower intracellular calcium, but that this effect was not sufficient to antagonise the contractile effects of phenylephrine or KCl. It should be noted, however, that both types of contractions could be relaxed by verapamil or hydralazine. The concentration of phenylephrine used in our experiments was submaximal, and phenylephrine-induced contractions could be almost completely blocked by pretreatment of the muscles for 30 s with relatively low concentrations of verapamil (Fig. 1). Cyclic GMP levels were not significantly altered by the verapamil pretreatment. Levels of the cyclic nucleotide were 15.8 ± 2.6 pmol per g tissue in the muscles exposed to phenylephrine alone and 11.3 ± 1.8 pmol per g tissue in muscles exposed to both verapamil and phenylephrine ($N=6$). Conversely, increases in cyclic GMP levels even larger than those shown in Table 1 were seen when the muscle segments were pretreated with sodium nitroprusside for 30 s before adding the phenylephrine. In these conditions cyclic GMP levels in the muscles exposed to both sodium nitroprusside and phenylephrine were increased more than 15-fold over the phenylephrine controls (from 15 ± 5 to 226 ± 27 pmol per g tissue, $N=3$). In spite of this large increase in cyclic GMP levels no detectable change in the phenylephrine-induced contractions was seen (Fig. 1). If cyclic GMP is in fact a mediator of relaxation, such a large increase in tissue levels of cyclic GMP should have at

least partially reduced the contractions caused by these submaximal concentrations of phenylephrine.

Our results indicate that increases in cyclic GMP levels are probably not responsible for the relaxant effects of sodium nitroprusside, hydralazine or verapamil in rat vas deferens. Further work is needed to clarify the role of cyclic GMP in smooth muscle relaxation.

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Arginine-specific reagents remove sodium channel inactivation

PROTEINS are often suggested to be the molecular components of excitable membranes which confer voltage-dependent permeability properties on nerve and muscle cells. Because of the relatively low concentration of the protein molecules directly responsible for ionic conductances compared with other membrane components, the isolation, characterisation, and reconstitution of these proteins is still at a rather early stage. Certain aspects of the molecular nature of the conductance mechanisms of excitable tissues, however, can be deduced using chemical reagents which specifically modify protein molecules. In particular, the sodium inactivation process can be eliminated by treatment with various proteolytic enzymes^{1–3,6}. Of these, trypsin is probably the most selective, cleaving mainly at arginyl or lysyl residues⁴. Several investigators have suggested that just such an exposed positively charged residue might be responsible for blocking the sodium conductance pathway to produce inactivation^{5,6}. Therefore, it should be possible to alter or remove inactivation by changing the configuration and/or charge of the blocking residue with a reagent specific for the residue involved. Glyoxal, phenylglyoxal and condensed 2,3-butanedione are three such reagents which are very reactive with the guanidino group of arginine^{7–9}. This paper describes the effect on sodium inactivation of these agents when they are internally perfused in the squid axon.

Two methods of internal perfusion were used: the 'roller' method¹⁰, and the 'cannula' method¹¹. In either case, axons were then mounted in a plexiglass chamber designed for voltage clamping using conventional techniques^{12,13}. Feedback compensation was used in all experiments to reduce errors due to membrane series resistance.

Glyoxal was allowed to react with the inner surface of the axonal membrane by perfusing the fibre for 10–15 min with a 5 mM solution of the trimeric dihydrate in an internal solution at pH 9.0. The pH 9.0 internal solution by

itself produced only a slight reduction in peak inward sodium current, with little (if any) effect on inactivation as estimated from the steady-state inward current at the end of a 10-ms pulse. At the end of the reaction period, the glyoxal had reduced the peak inward current to approximately 50% of its original control value, but the steady-state current at the end of the 10-ms pulse had increased by 200%. This result seems to reflect the complete removal of inactivation in affected sodium channels, as the time constant of decay in the component of current that still inactivated was unaffected by glyoxal treatment. Figure 1 shows the effect of glyoxal treatment.

To quantify this effect, a 25–50-ms conditioning prepulse was used to determine the relationship between inactivation and membrane potential before and after glyoxal treatment. In Fig. 2*b*, the normalised peak inward current is plotted against conditioning potential. In the control case, the peak inward current is rapidly reduced as the conditioning prepulse becomes more depolarised until, for large prepulses, more than 90% of the peak inward current is inactivated. After treatment with glyoxal, a portion of the peak sodium current was inactivated with a voltage dependence similar to the control, but a large fraction of the current is unaffected by the conditioning prepulse even at large depolarisations. We associate this fraction with the sodium inactivation molecules that have reacted with glyoxal.

Fig. 1 Current-time records before and after glyoxal. *a*, Family of current time records in the control solution: 500 mM CsF, 5 mM NaF and 20 mM CHES buffer adjusted to pH 9.0. The external solution was filtered seawater. The currents were produced by potential steps in increments of 10 mV from -30 mV to +90. *b*, Effect of 5 mM of the trimeric dihydrate of glyoxal (Sigma) applied in the control solution for 15 min. The number of voltage steps has been reduced for clarity. The currents shown are produced by potential steps in increments of 10 mV from -10 mV to +40. The experiment was carried out at the Marine Biological Laboratory, Woods Hole, Massachusetts, on axons of *Loligo pealei*.

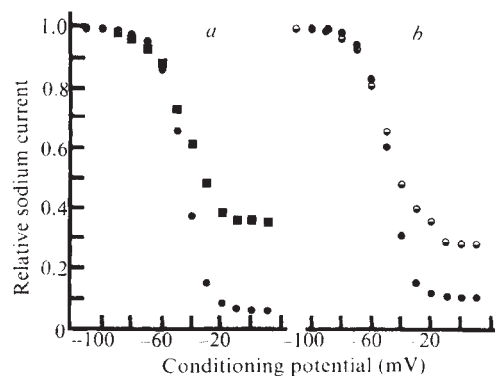
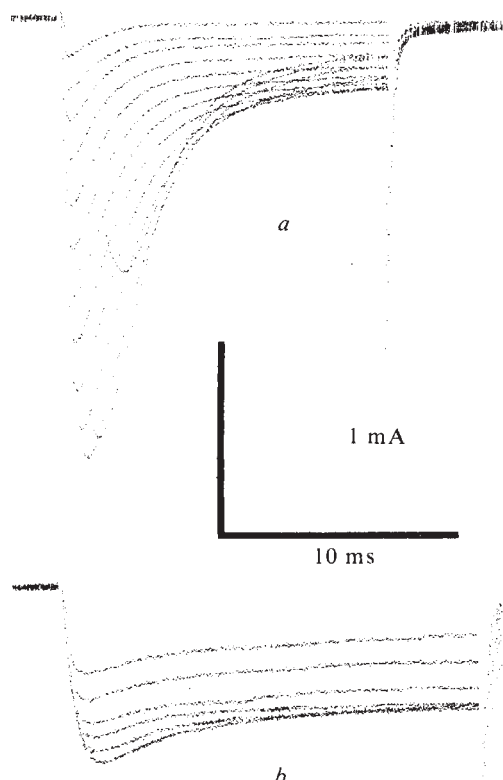


Fig. 2 Removal of inactivation by arginine-specific reagents. Thirty millisecond conditioning prepulses of various amplitudes were given before a test pulse (+10 mV). The ratio of the test current for different conditioning potentials to the maximum test current at large negative conditioning potentials is plotted. *a*: ●, control values with an internal solution containing 400 mM KF, 100 mM TEABr, 5 mM NaF and 20 mM CHES buffer adjusted to pH 9.0; ■, same conditions after treatment with 15 mM diacetyl trimer for 45 min. *b*: ●, control internal solution was 500 mM, CsF, 5 mM NaF and 20 mM CHES buffer to pH 9.0; ○, same conditions after treatment with 5 mM of the trimeric dihydrate of glyoxal. The trimer of 2, 3-butanedione was synthesised according to the method of Grossberg and Pressman¹⁴. A 15% aqueous solution of 2, 3-butanedione buffered to pH 8.0 with potassium phosphate was allowed to condense at constant pH for 48 h before use. This yields a solution of approximately 0.3 M trimer.

We also applied phenylglyoxal. A 1 mM solution of the reagent applied in a pH 7.3 internal saline produced a decrease in peak inward current without any removal of inactivation. Higher concentrations of phenylglyoxal led to a rapid and relatively complete removal of peak inward current, and a concomitant increase in leakage. Effects on inactivation in these conditions were difficult to determine.

Use of the third arginine reagent, 2,3-butanedione trimer, is complicated by the long reaction times necessary at pH 7.3. Fortunately, reaction at higher pH is more rapid^{9,14}. In squid, perfusing with a 15 mM solution of trimer in internal perfusate at pH 9.0 led to a 50% reduction in peak inward current and substantial removal of inactivation (Fig. 2*a*).

None of the effects of application of the reagents is reversible. The reduction of peak inward current for all the reagents and the removal of inactivation for glyoxal and the diketone trimer were not altered even after repeated changes of internal perfusion solution free of the reagents.

The previous results made arginine (or possibly lysine) a likely component of the Na⁺ inactivation mechanism. To test the possibility that arginine might actually be the blocking residue of the inactivation molecule, we completely removed inactivation by treatment with Pronase², and then

Table 1

Amino acid	Effect	Fraction of sodium* current after treatment
Arginine	+	0.76 ± 0.07 (<i>n</i> = 6)
Lysine	0	0.96 ± 0.06 (<i>n</i> = 6)
Aspartic acid	0	1.00 (<i>n</i> = 1)
Glutamic acid	0	1.00 (<i>n</i> = 1)
Glycine	0	1.03 ± 0.06 (<i>n</i> = 3)
Histidine	0	1.08 ± 0.07 (<i>n</i> = 4)
Tyrosine†	0	1.01 (<i>n</i> = 2)
Asparagine	0	0.99 (<i>n</i> = 2)
Glutamine	0	0.99 ± 0.07 (<i>n</i> = 4)

Internal solution contained (mM): CsF 500, HEPES 20, Na⁺ + amino acid 50. External solution was filtered seawater.

*Ratio of inward current in the presence of the amino acid to the inward current in the absence of the amino acid (for a voltage step to 0 mV).

†Tyrosine was applied as a 5 mM solution.

internally applied various amino acids. Table 1 shows that of the amino acids tested only arginine shows a significant blocking ability. Notably, lysine produces no significant blocking effect. We feel that the reason arginine produces a simple blockage of current rather than a time-dependent reduction of current similar to normal inactivation is due to the relatively mobile character of the free amino acid. This blockage is analogous to the effect of tetraethylammonium (TEA) and its higher analogues on the sodium channel⁶. TEA equilibrates with the sodium conductance mechanism so rapidly that no time dependence is observed. TEA analogues with a hydrophobic side chain equilibrate at a much slower rate, producing a time dependent reduction in current.

With this analogy in mind, the possibility that arginine could be the blocking residue was tested further by internally applying the polypeptide, polyglycyl arginine amide,

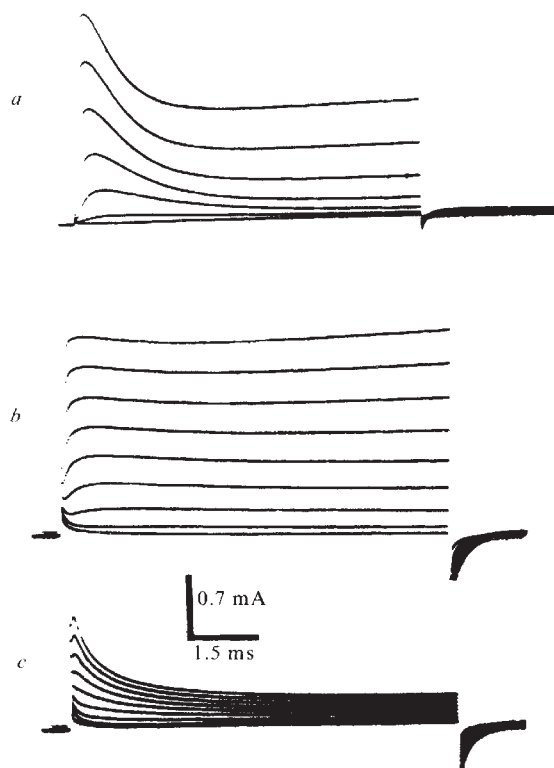


Fig. 3 Effect of polyglycyl-*N*-acetyl arginine amide on outward sodium currents in a fibre, without inactivation. Axon bathed in Tris-HCl 450 mM, MgCl₂ 50 mM, CaCl₂ 10 mM, pH 7.9; internally perfused with 250 mM NaF, 500 mM sucrose, 5 mM Tris-HCl, pH 7.3; temperature 5 °C. The figure shows currents in response to voltage clamp pulses that took the membrane potential to the values indicated below from a holding potential of -60 mV. *a*, Current records for pulses to -30, -10, 10, 30, 50, 70 and 90 mV. *b*, Current records after perfusing the axon for 12 min with Pronase dialysed overnight against EGTA (final concentrations: Pronase, 1 mg ml⁻¹, EGTA 1 mM). Membrane potential during the pulses: -30, -10, 10, 30, 50, 70, 90, 110 and 130 mV. *c*, Seven minutes after the records in *b* were taken, the axon was internally perfused with polyglycyl-*N*-acetyl arginine amide (approximately 0.1 mM molecular weight 10,000) and the records shown taken 5 min later. Membrane potential during the pulses: -30, -10, 30, 50, 70, 90, 110 and 130 mV. The polyglycyl-*N*-acetyl arginine amide was synthesised by coupling polyglycine of molecular weight 5,000 to 10,000 (Sigma) with *N*-acetyl-arginine (Sigma) using a water-soluble carbodiimide as a coupling agent¹⁵. Separation of the desired peptide from unreacted components was accomplished by chromatography in Sephadex G-10, followed by ion exchange in CM-Sephadex. The experiment was carried out using axons of *Loligo forbesi* at the Marine Biological Association, Plymouth, UK. Similar results were obtained with a longer polyglycyl arginine derivative (molecular weight 20,000).

in an axon which had previously been treated with Pronase to remove inactivation. The results suggest that this peptide can mimic sodium inactivation (Fig. 3).

In axons treated with Pronase, perfused internally with sodium fluoride containing the peptide and bathed in sodium-containing artificial seawater, time dependent block of both the inward as well as the outward sodium currents was observed (E. Rojas and B.R., unpublished).

When considering these results, special attention must be given to the specificity of the reagents used to react with the presumptive arginyl residue. For each reagent used, the only significant reaction besides that with guanidino moieties is with amino groups. The reaction of all the reagents with amino groups is much slower and less complete than with arginine⁷⁻⁹.

Thus, the reaction kinetics of the reagents make reaction with lysine seem less likely than arginine. If the reagents are actually attacking a blocking residue acting as either a physical or electrostatic 'plug' during inactivation, the results of the amino acid and polypeptide application make the reaction with arginine even more attractive since lysine seems to be unable to block the sodium channel.

The reduction in the peak inward current after application of the reagents may be due to several things. Of course, the reagents may be directly reacting with the sodium conductance mechanism in some unspecified way. Alternatively, each of the reagents may be capable of cross-linking reactions¹⁶. This might lead to an occasional reaction where the blocking residue of the inactivation molecules is cross-linked while in a blocking position, with a consequent reduction in sodium conductance.

We can consider these results in the context of the models already suggested^{5,6}. Both models require a charged group that is 'tethered' to the membrane. The charged group is capable of blocking open sodium channels and consequently producing inactivation. In the model of Rojas and Rudy, it is strongly suggested that the charged moiety is an arginyl residue in a peptide chain. Our data with the amino acids and polypeptide suggest that an arginyl residue could actually be the blocking component, but that lysine could not.

But, whatever the actual mechanism of inactivation, our results with glyoxal and diacetyl trimer strongly suggest that an arginine residue must be an important functional part of the inactivation process.

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Photoreceptor electric potential in the phototaxis of the alga *Haematococcus pluvialis*

THE nature of the primary processes in the photoreceptor and the mechanism of their coupling with the mechanical response of the flagella are the most important and, at the same time, the least investigated problems of the phototaxis in flagellates^{1–5}. It has been suggested that the transduction of the light stimulus in phototaxis might involve bioelectric processes^{4, 6–9}, but no direct experimental evidence supports this hypothesis. The bioelectric control of the locomotor responses to light^{10, 11}, mechanical¹² or electrical¹³ stimulation has been found in ciliates, but they do not possess the orientated phototactic response mechanism¹⁴ of flagellates. Now we demonstrate the photoinduction of electric potentials related to phototaxis in the unicellular flagellated alga *Haematococcus pluvialis*. Photo-stimulation evokes a graded receptor potential which gives rise to a regenerative potential dependent on the presence of extracellular calcium ions.

Insertion of a microelectrode rapidly damages the cell, hence electrical potentials were registered extracellularly. In the developed technique the cell was sucked (under microscopic control) into a tip of a glass micropipette (Fig. 1), causing two parts of the cell surface, the inside and outside, to become electrically isolated by the glass of the pipette (shunt resistance, up to 100 MΩ) thus enabling measurement of the electrical potential difference (PD) between them. The micropipette and the chamber in which its tip was located were filled with the same solutions (usually growth medium) and connected by KCl bridges and calomel electrodes to the amplifying and registering equipment similar to that described earlier^{15, 16}. A signal was considered positive if the inner part of the pipette had a positive potential in reference to the potential of the chamber. The time resolution of the system was about 1 ms.

For actinic illumination, a flash xenon lamp (flash duration, less than 1 ms) or a halogen incandescent lamp provided with an electrical shutter (opening time, about 1 ms) were used. The intensity of a light stimulus was measured by a thermopile or a calibrated photodiode and was changed from 10^2 to 10^6 erg cm⁻² s⁻¹ by neutral light filters. The measurements were carried out at room temperature using a water infrared filter.

The cells of *H. pluvialis* were grown at 20°C under constant illumination with fluorescent lamps (500 lx) in a medium containing 1 mM KNO₃, 0.4 mM K₂HPO₄, 0.3 mM MgSO₄, 0.3

mM Ca(NO₃)₂, and a standard solution of trace elements, and 3–7-d-old cultures were used.

The sucking of a cell into a micropipette leads to its slight deformation, but even after a 2–3-h experiment the cell regained its normal motility and a phototactic ('stop') reaction after being released from the pipette. In the dark or under red light illumination, there is no PD between the chamber and the inside part of the micropipette separated by the cell. On 1 ms flash illumination with white or blue-green light, a fast transient PD between the two parts of the cell surface (inside and outside the pipette) is generated (Fig. 2a). This primary photoinduced potential difference, PPD, comprises a fast rise of PD and its slower drop to zero. The response is graded, both the amplitude and the rate of the potential changes rising with the increase of light intensity.

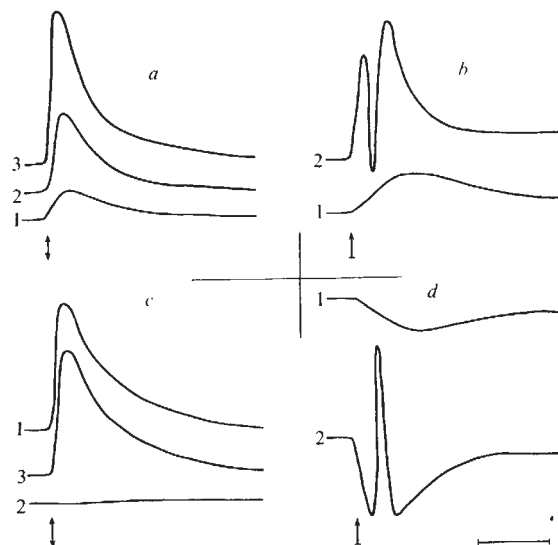
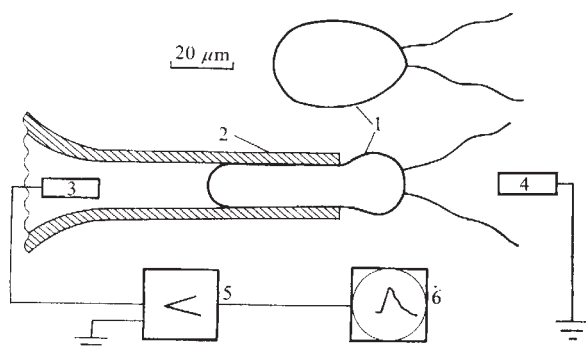


Fig. 2 Kinetics of photoinduced electric potential difference (PD) between the two parts of the cell surface, the inside and the outside of the pipette. The arrows mark the moment of a flash (for *a* and *c*) or of a switching on of continuous light (for *b* and *d*). An upward movement of the traces means an increase in potential inside the micropipette. Calibration: 2 mV and 100 ms. *a*, The posterior part of the cell is inside the pipette for *a*, *b*, *c*. Primary PD induced by 1 ms flash of three different intensities (1, 5 and 12 relative units for curves 1, 2 and 3, respectively). *b*, PD induced by continuous light of different intensity (1 and 8 relative units for curves 1 and 2, respectively). At the intensity exceeding a threshold level, primary PD becomes superimposed by a secondary regenerative response (curve 2). *c*, Effect of 3 mM azide on photoinduced PD. 1, Before the addition of azide; 2, 30 s after azide addition; 3, after the removal of azide from the chamber by a fresh solution. *d*, The same as *b*, but the anterior part of the cell is inside the pipette.

Fig. 1 Scheme of a cell fixation in a suction micropipette and extracellular potential difference registration. 1, Cell; 2, tip of the micropipette; 3, calomel electrode connected to the interior of the micropipette; 4, calomel electrode connected to the chamber; 5, amplifier; 6, oscilloscope.



When the intensity and/or the duration of a light stimulus exceed some threshold, the PPD becomes superimposed by a regenerative electric response (RR) (Fig. 2b). This spike-like signal has a two-wave form and usually one of the waves (positive or negative) dominates. Its amplitude reaches a constant level within a narrow range of rising stimulus intensities. Further light intensity increase leads to the shortening of the lag-period of RR from 60 ms to 5 ms. The blue-light induced RR has a refractory period of about 0.5–1.0 s. The appearance of the blue-light induced spike can be stimulated by the red background illumination or by the increase of the frequency of blue flashes (from 1 to 20 per min), while the prolongation of a dark period leads to the disappearance of RR. These features of the spike-like electrical signal allow its separation from PPD.

RR is sensitive to poisoning by heavy metals or to the removal of Ca²⁺ ions by EDTA. Both factors also affect PPD, but to a much lesser degree. Spontaneous spikes can be sometimes

observed in the dark or under constant illumination. Their complex kinetics is similar to that of the photoinduced signals.

We believe that RR reflects the excitation of the whole cell membrane in a similar way, perhaps, to that found in ciliates^{11,13}. It is noteworthy that removal of Ca^{2+} also blocks the phototactic response (stopping, reversed locomotion) of *Chlamydomonas*⁹. Thus the RR is likely to play a significant part in phototaxis.

PPD has no (or less than 1 ms) lag-period. Its initial rise rate depends linearly on the light intensity up to $10^6 \text{ erg cm}^{-2} \text{ s}^{-1}$, the rise time being not more than 5 ms at this intensity. In contrast to this, the amplitude of the signal has a nonlinear light dependence and a tendency to saturate at high intensities. The PPD signals can reach 5–7 mV and can be reversibly inhibited by azide (Fig. 2c).

It was found that the sign of PPD is determined by the orientation of the cell in the pipette. The positive signals were registered in all cases when the posterior or lateral part of the cell was sucked into the pipette (Fig. 2a, b, c). If the same cell was fixed in the pipette so that the anterior, flagella-bearing part was in the pipette, signals of negative polarity but similar form were observed (Fig. 2d). This observation suggests that the anterior zone of the cell becomes negatively charged with respect to the other part of the cell surface under the action of light.

It is natural to suggest that the photoinduced polarisation of the cell is due to the asymmetrical location of the photoreceptor (an electric potential generator) in the cell membrane. Taking into account that the negative potential can be registered only from a small part of the anterior cell surface, one may conclude that the photoreceptor is located in this part of the cell, has a small size and generates the positive potential inside the cell with respect to the outer space under the influence of light. A simple equivalent scheme of the extracellular measurements of the potential can be proposed (Fig. 3). The scheme does not have capacity elements and cannot be used to describe the kinetics of the signals.

The anterior (a) and posterior (p) parts of the cell with transmembrane resistances R_a and R_p , respectively, are externally separated by the shunt resistance R_{sh} . The resting potentials (E_m) of the anterior and posterior membranes compensate one another in the dark. On illumination, the photoreceptor in the anterior part of the cell membrane generates an electrical potential E_r which is measured across R_{sh} as the PD. E_r can be determined if R_{sh} , PD and R_p are known. It was found that the light-induced voltage rises linearly with increase in shunt

Fig. 3 Simple equivalent scheme of the extracellular registration of the transmembrane receptor potential (see text). p and a, Posterior and anterior parts of the cell surface (inside and outside the pipette); R_p and R_a , the resistances of the posterior and anterior parts of the cell membrane; E_m , transmembrane potential in the dark; E_r , photoinduced receptor potential; R_{sh} , shunt resistance between p and a; G, voltmeter.

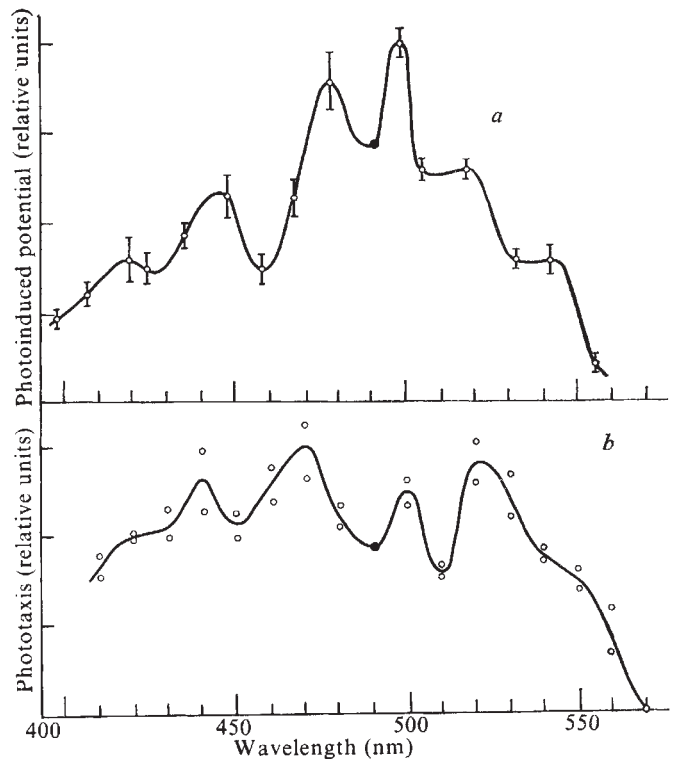
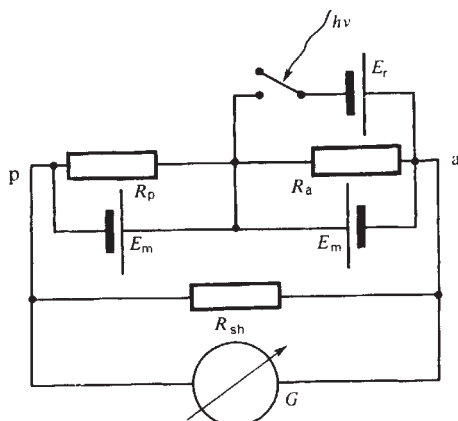


Fig. 4 Action spectra for the initial rate of PD rise (a) and the rate of phototactic cell accumulation in the lighted region (b). Spectral sensitivity at each wavelength was compared to that at 490 nm. Each point on curve a represents a mean value of 6–12 measurements $\pm \sigma$ corrected for the number of incident quanta. Spectrum b was measured under the conditions of equal no. of incident quanta at each wavelength.

resistance (measured as a difference between the resistance of the pipette with the cell and that without the cell) as the cell is sucked into the pipette. With R_{sh} of $10^8 \Omega$, PD is 3.5 mV. Assuming that the specific membrane resistance of the cell membrane has an average value of about $2 \times 10^4 \Omega \text{ cm}^2$ (refs 17, 18), R_p is $1.4 \times 10^9 \Omega$ for a spherical cell 30 μm in diameter. For these values, the light-induced transmembrane potential E_r is about 50 mV. Because of the changes in the geometry of the cell in the pipette and of the possible deviations of the value of the specific membrane resistance from the accepted average, this calculation of E_r is a rough estimate.

It was found that only the blue-green region of the spectrum is efficient in the generation of the described potentials. The red and infrared light ($\lambda > 630 \text{ nm}$) have no effect, even at the intensities 3–4 orders of magnitude higher than the usually effective intensities of the blue light. The action spectrum for the rate of the PPD rise was measured with the use of interference filters (band pass, 10 nm) and was compared to the action spectrum of phototaxis of this organism (Fig. 4). For the net phototaxis action spectrum measurements, the equipment similar to that described earlier by Lindes *et al.*¹⁹ was used. The kinetics of the cell accumulation in the actinic zone was monitored by a testing (measuring) beam ($\lambda = 750 \text{ nm}$). The intensity of the monochromatic actinic light at each wavelength (band pass, 5 nm) was measured and equalised in a number of incident quanta before each kinetic curve was recorded. The initial rate of the cell accumulation in the illuminated zone was taken as a criterion of phototaxis (phototactic index)²⁰. As seen from Fig 4, the action spectra of the PPD generation and the net phototaxis are similar. This points to the generation of the described potential by the receptor pigment(s) of phototaxis.

Rapid (less than 30 s) and reversible inhibition of the electrical signal by azide (3 mM), known as an efficient and reversible inhibitor of phototaxis²¹, also indicates that there is

close connection between the potential generation and phototaxis. PPD, however, is not sensitive to 3-(3,4-dichlorophenyl)-1,1-dimethylurea, the inhibitor of photosynthesis, in usually efficient concentrations (10^{-5} M).

The time characteristics of the electrical signal, its dependence on the intensity and spectral composition of the actinic light, the localisation of the photoreceptor, the character of the inhibitors' action, and others suggest that the photoinduced potential is a photoreceptor potential of phototaxis. The mechanism of the potential generation is not yet clear, and may be based on changes in permeability for ions, as well as active electrogenic processes. Also, the receptor potential may affect the action of flagella directly or through the secondary regenerative response. To solve these problems further experiments are necessary.

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Stimulation of brain membrane protein phosphorylation by calcium and an endogenous heat-stable protein

THE important role of Ca^{2+} in the physiology of the nervous system is well documented^{1,2}. However, the biochemical mechanisms underlying certain of its physiological effects, such as stimulus-secretion coupling^{3,4} and synthesis of catecholamines^{5,6}, have not yet been elucidated. Calcium has been implicated in several biochemical reactions of potential importance to synaptic function. Thus, calcium and a heat-stable calcium-binding protein activate the cyclic nucleotide phosphodiesterase from mammalian brain^{7,8}. The calcium-dependent regulator (CDR) from porcine brain⁹, bovine heart¹⁰ and bovine brain¹¹ has been purified and characterised. CDR seems to be a calcium receptor as it binds calcium strongly and specifically. There is evidence that a $\text{CDR} \cdot \text{Ca}^{2+}$ complex is the true activator of cyclic nucleotide phosphodiesterase¹²⁻¹⁴. A detergent-solubilised preparation of brain adenylate cyclase can also be activated by this calcium-binding protein¹⁵. Calcium stimulates protein phosphorylation in both intact^{16,17} and lysed^{18,19} synaptosomes and this cyclic nucleotide-independent mechanism may mediate or modulate some of the intracellular effects of Ca^{2+} on the function of presynaptic nerve terminals. We report here that calcium-dependent phosphorylation of synaptosomal membrane fractions from rat cerebral cortex requires an endogenous protein factor present in the synaptosomal cytoplasm. Calcium stimulated phosphorylation is lost on purification of synaptic membranes and can be effectively recovered by reconstitution with either the synaptosomal cytoplasm or with a purified preparation of CDR. Thus, regulation by calcium of calcium-dependent protein kinase activity

may be mediated physiologically by the calcium-binding protein postulated to regulate cyclic nucleotide phosphodiesterase and adenylate cyclase.

The effect of calcium on endogenous protein phosphorylation in lysed synaptosomes and in synaptic membranes is shown in Fig. 1. Synaptosomes obtained from rat cerebral cortex were lysed by hypo-osmotic shock and assayed for incorporation of ^{32}P -phosphate from $\gamma\text{-}^{32}\text{P}$ -ATP into protein (Fig. 1, lanes 1 and 2). Calcium caused a marked stimulation of endogenous protein phosphorylation. A net increase in ^{32}P -phosphate incorporation into a number of protein bands was observed, with those having molecular weights of about 51,000 and 62,000 showing highest levels of incorporation. Similar patterns of stimulation by calcium have been observed by DeLorenzo and Freedman^{19,20}, though much higher calcium ion concentrations seem to be required in their system.

The calcium-dependent phosphorylation observed in assays containing both synaptic membrane fractions and synaptosomal cytoplasm (that is, lysed synaptosomes) is lost on

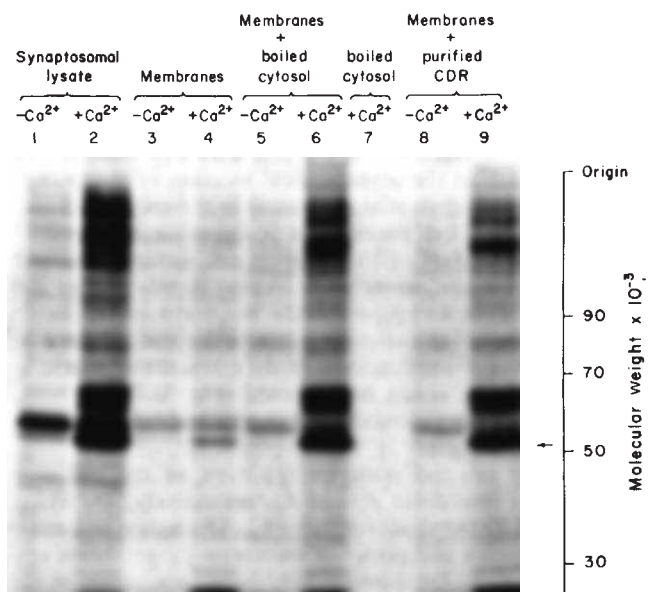


Fig. 1 Effect of Ca^{2+} , synaptosomal cytoplasm, and purified CDR on endogenous protein phosphorylation of brain membranes. Male Sprague-Dawley rats (150-200 g) were killed by decapitation and the cerebral cortex used for preparation of synaptosomes (P2) by minor modification of the method of Gray and Whittaker²⁸. The suspension of synaptosomes obtained from 2 g of cerebral cortex was subjected to osmotic shock by homogenisation in 10 volumes of ice-cold water (15 ml) and kept on ice for 30 min to optimise lysis. The synaptosomal lysate contained 2.5 mg protein ml^{-1} . Total membranes were obtained from lysed synaptosomes by centrifugation at $150,000g$ for 30 min. The released synaptosomal cytosol (0.7 mg ml^{-1}) was removed, heated at $95-100^\circ\text{C}$ for 5 min, and used as the source of activator. The pellet was resuspended in 15 ml of 5 mM Tris-HCl (pH 7.0), centrifuged as above, and again resuspended in 15 ml of 5 mM Tris-HCl (pH 7.0). This resuspended pellet (1.7 mg ml^{-1}) served as the source of activator-deficient membranes. Membranes stored at -20° were stable for about 1 month. CDR was purified to homogeneity by the method of Teo *et al.*¹⁰. The reaction mixture for assay of endogenous protein phosphorylation (final volume, 100 μl) contained: 50 mM PIPES buffer (pH 7.0), 10 mM MgCl_2 , 1 mM dithioerythritol, 0.2 mM EGTA (minus calcium) or 0.2 mM EGTA + 0.5 mM CaCl_2 (plus calcium), 5 μM $\gamma\text{-}^{32}\text{P}$ -ATP ($3.0-7.0 \times 10^4$ c.p.m. pmol^{-1}), and, where indicated, 50 μg lysed synaptosomes, 34 μg membranes, 14 μg boiled cytosol and 0.25 μg CDR. The reaction was initiated by addition of the $\gamma\text{-}^{32}\text{P}$ -ATP after preincubation for 30 s at 30°C . Incubation was carried out for 10 s, the reaction terminated by addition of 50 μl of an 'SDS-stop solution', and a 75 μl aliquot of the sample analysed by SDS-polyacrylamide gel electrophoresis and autoradiography as described previously²⁹. Using procedures described elsewhere¹⁷, radioactive bands were found to be due to incorporation of ^{32}P -phosphate into protein (by phosphomonoester linkage) rather than into lipid or nucleic acid. Results similar to those shown here were obtained when more purified preparations of synaptosomes were used.

preparation of cytoplasm-free membranes (Fig. 1, lanes 3 and 4). This phenomenon was not due to inactivation of the calcium-dependent kinase activity during experimental manipulations as it could be regained by addition of an amount of synaptosomal cytoplasm (Table 1) or boiled synaptosomal cytoplasm (Fig. 1, lanes 5 and 6; Table 1) commensurate with the amount of membrane protein present. The heat-stable factor in the cytosol which conferred calcium-dependent phosphorylation on washed membranes did not exhibit any endogenous phosphorylation (Fig. 1, lane 7), nor did it show any histone, protamine or casein kinase activity (data not shown).

The heat stability of the stimulating factor in the cytosol suggested a possible relationship to the calcium-binding protein that modulates activities of cyclic nucleotide phosphodiesterase and adenylate cyclase. Therefore, this calcium-dependent regulator was purified to homogeneity from bovine cerebral cortex by the method of Teo *et al.*¹⁰, in order to test its effect in the calcium-dependent protein phosphorylation system. Addition of this protein to washed synaptosomal membrane fractions restored calcium-dependent protein phosphorylation (Fig. 1, lanes 8 and 9). The pattern of phosphorylation obtained on addition of heat-treated synaptosomal cytoplasm was indistinguishable from that obtained on addition of CDR, suggesting that the two factors are functionally equivalent.

Stimulation of protein phosphorylation was dependent on the presence of both calcium and either the cytosol factor or CDR. Thus, calcium in the absence of boiled cytosol and boiled cytosol in the absence of calcium were ineffective, whereas addition of both calcium and boiled cytosol restored phosphorylation (compare lanes 3, 4, 5 and 6). Analogous results were obtained with CDR (compare lanes 3, 4, 8 and 9). The endogenous activator, like CDR, is extremely heat stable, non-dialysable, resistant to DNase and RNase, and sensitive to trypsin (Table 1).

CDR is present in synaptosomal cytosol of bovine and rat cerebral cortex and should, therefore, account for some of the activation seen by reconstituting membranes with synaptosomal cytosol. However, this does not exclude the possibility that other factors also present in the cytosol may be physiologically important regulators of the calcium-dependent phosphorylation reaction. Therefore, we have recently purified a protein from bovine cerebral cortex based on its ability to stimulate protein phosphorylation in activator-depleted membranes (in preparation). Only a single peak of activity was present throughout the purification procedure. This activity co-purified with the phosphodiesterase activator, suggesting that this endogenous

activator of the calcium-dependent phosphorylation system is CDR.

The calcium-dependent regulator seems to have a rather broad distribution in the animal kingdom²¹. Various mammalian and invertebrate tissues have been found to contain low levels of calcium-activatable phosphodiesterase and very high levels of CDR²¹⁻²⁶, leading many investigators to speculate that there must be other functions for this protein. Indeed, Kretsinger has postulated that all of the processes in which calcium functions as a second messenger are mediated by calcium-binding proteins with binding properties similar to those of CDR²⁷. The findings reported here demonstrate that calcium and CDR can affect protein phosphorylation directly, by stimulation of a calcium- and CDR-dependent protein kinase system, and suggest that this system may mediate some of the presynaptic actions of calcium. Calcium and CDR can presumably also regulate protein phosphorylation indirectly, by changing cyclic nucleotide levels by means of effects on cyclic nucleotide phosphodiesterase and adenylate cyclase activities.

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Table 1 Characterisation of the cytosol factor

Addition to activator-depleted membranes	³² P-phosphate incorporated % of control
None	27
Untreated cytosol	100
Heat-treated cytosol	95
Dialysed cytosol	99
DNase-treated cytosol	99
RNase-treated cytosol	97
Trypsin-treated cytosol	28

Cytosol (14 µg) was preincubated in the presence of 50 mM Tris-HCl (pH 7.2), 1 mM MgCl₂ and, where indicated, DNase (50 µg ml⁻¹), RNase (50 µg ml⁻¹) or trypsin (10 µg ml⁻¹), in a total volume of 30 µl, for 60 min at 30 °C. Trypsin inhibitor (egg white) was then added at a final concentration of 25 µg ml⁻¹ to the trypsin treated sample. The cytosol factor remaining after these treatments was assayed for its ability to support endogenous protein phosphorylation of synaptic membranes (34 µg) using the reaction mixture described in the legend to Fig. 1. For quantitative determinations, the radioactive band at a molecular weight of 51,000 (see arrow, Fig. 1) was localised by autoradiography, cut out of the dried gel and counted by liquid scintillation spectrometry. Incorporation in the presence of untreated cytosol is taken as 100% and represents 17.3 pmol phosphate per min per mg membrane protein. DNase, RNase and trypsin + inhibitor (egg white) added to synaptic membranes without cytosol had no effect. Results qualitatively similar to those shown here were obtained with several other proteins bands.

The use of *Xenopus* egg cells to assay the mRNA of single cells

TRANSLATION of mRNA in egg cells and oocytes of *Xenopus laevis* Daudin has been shown to be very efficient for mRNAs from a variety of cells¹⁻⁷ and has uses in developmental biology, molecular biology and immunology⁸. We have also reported its use in cancer research⁹. One of the main disadvantages of this tool, however, is the fact that fairly extensive RNA extraction and purification procedures are required. In particular, in cases when different cell types are mixed, as in the case with immunologically active cells, it is almost impossible to obtain information about the activity of the separate cell types. Studies in this field are always dealing with, at best, highly enriched cell populations. Therefore we have looked for a method which avoids RNA extraction and purification, with the aim of being able to work with separate cells. We have tried to inject cell homogenates

directly into egg cells of *Xenopus*, and in the experiments reported here, we chose myeloma cells (LOU/M/WS1 rats with myeloma IR2 synthesising immunoglobulin E), because we could analyse the translation products easily and we could start with a relatively homogeneous cell population. Our results suggest that it is possible to study the translation products of single myeloma cells using this technique.

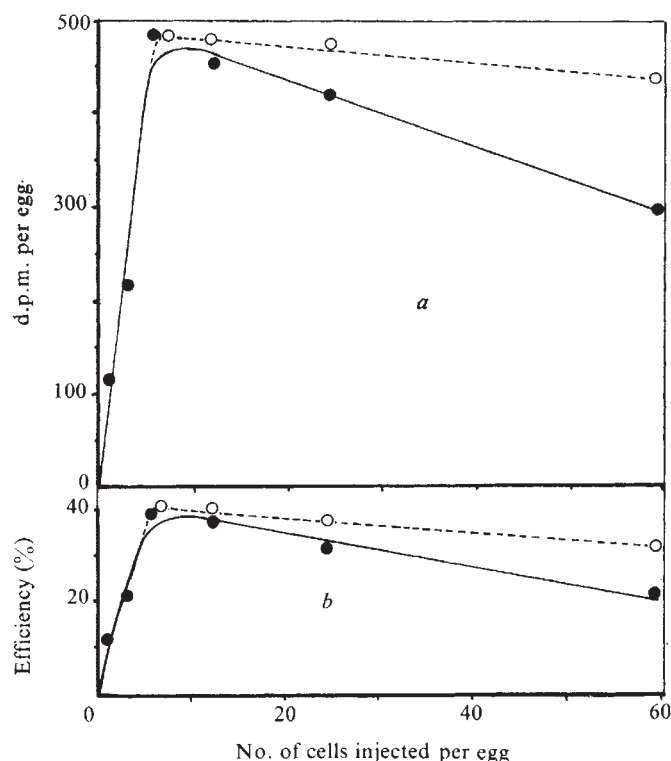


Fig. 1 *In ovo* synthesis of rat IgE. Female LOU/M/WS1 rats were infected with 0.5 ml myeloma IR2 cells (IgE-secreting myeloma¹⁰). After 7–9 d ascites was tapped and on average it contained 12.6×10^6 cells per animal. These were diluted 1:1 with Earle's balanced salt solution and layered on top of 5 ml Ficoll–Isopaque ($d=1.078$). After centrifugation for 30 min at 1,500g at room temperature¹¹, lymphocytes were taken from the top of the Ficoll–Isopaque cushion and washed twice with Earle's BSS. After the second washing, the cell suspension was divided into two equal parts, one for direct injection and one for RNA extraction. Both parts were frozen in solid CO₂ and used for injection and extraction the next day. Obtaining and handling of the egg cells of *Xenopus laevis* was as described earlier⁷, the only difference being the absence of actinomycin D in the incubation medium. Myeloma cells were suspended in double-concentrated injection medium, counted and homogenised. The final cell concentration corresponded to 5×10^6 cells ml⁻¹. One aliquot was diluted 1:1 with ³⁵S-methionine (Radiochemical Centre, Amersham, UK; specific activity 25 mCi mmol⁻¹) and 50 nl injected, containing 3×10^5 d.p.m. = 5 pmol methionine. A number of dilutions was then made of the cell homogenate (1:2, 1:4, 1:10, 1:20, 1:40, 1:125). Of each sample, 50 nl was injected per egg cell after 1:1 dilution with radioactive precursor. As a control, 50 nl of 1:1 diluted precursor was injected alone. RNA was extracted as described previously¹², yielding 0.16 ng total RNA per myeloma cell. Eggs were incubated for 16 h at 17 °C and homogenised in 400 µl PBS. The homogenate was taken up in a total of 800 µl PBS and centrifuged 30 min at maximum speed in a Spinco 50Ti rotor at 4 °C. To 100 µl of the clear supernatant 5 µl absorbed goat anti-rat IgE was added. After incubation at 37 °C for 15 min, 100 µl of rabbit anti-goat IgG was added and the mixture was incubated at 37 °C for a further 15 min. After overnight precipitation at 4 °C the pellets were spun down in an Eppendorf centrifuge 3200 and washed thrice with cold PBS. The final pellet was suspended in 100 µl of a buffer containing 0.1 M Tris-glycine pH 8.3, 2% SDS, 5 mM 2-2-mercaptoethanol, and counted on Whatman 3 MM filter paper disks after drying in a Nuclear Chicago Liquid Scintillation Counter (efficiency ~ 70%). *a*, Radioactivity precipitable with anti-IgE after subtraction of the control value (300 d.p.m.). ●, Disrupted cells injected; ○, Crude RNA injected. In addition to the specific precipitation, a total, 10% trichloroacetic acid (TCA) precipitate of every sample was counted. *b*, Efficiency of translation expressed as the ratio of d.p.m. precipitable with anti-IgE over d.p.m. precipitable with 10% TCA. All experiments were done in triplicate.

The result of injecting several dilutions of cell homogenate are given in Fig. 1. Comparison of these results with those obtained after injection of a cold phenol RNA preparation shows no difference in response at greater dilutions. We conclude, therefore, that *in ovo* synthesis of IgE continues at the same rate following injection of RNA or cell homogenate of equal cell numbers, up to a maximum at about ten cells. At higher concentrations, however, a large decrease in specifically precipitable radioactivity is found after injection of cell homogenate, whereas this amount is almost constant after injection of RNA. The decrease is not caused by a general inhibition of translation by substances in the cell homogenate—as confirmed by plotting the efficiency, expressed as the ratio of radioactivity precipitable with anti-rat IgE to TCA-precipitable radioactivity (Fig. 1*b*). Clearly, only the specific translation is inhibited at increasing cell number. Purified, deproteinised RNA does not show inhibition to this extent. Therefore, this phenomenon may depend on protein(s). The inhibitors do not interfere with the translation machinery itself, since the total TCA-precipitable radioactivity is unaffected. The results show the presence in the myeloma cell homogenate of a substance(s) that specifically inhibits the translation of injected RNA. The source of this inhibitory material is uncertain, but the fact that greater concentrations of cell homogenate are required to effect inhibition could indicate that it is derived from other than myeloma cells. Since our cell homogenate was prepared from cells in the ascites, it is also very likely to contain material from other cells. We did not determine which cell type could be responsible for the observed inhibition.

Maximum efficiency of translation is found after injection of the content of about ten cells. In another study with myeloma cells, saturation of the *Xenopus* translation machinery was found after injection of 0.9 ng A-rich-containing RNA⁸. If we assume that there was the same percentage of A-rich-containing RNA in the myeloma cells that we used, saturation in our experiments was reached after injection of comparable amounts of RNA.



Fig. 2 Electrophoretic analysis of the translation products of *a*, endogenous *Xenopus* mRNA and *b*, mRNA from IR2 myeloma cells. The position of unlabelled marker IgE (ref. 13), run in the same gel, is also indicated (*c*). Before electrophoresis, egg homogenates were treated with goat anti-rat IgE as indicated in Fig. 1. The precipitates were dispersed in a buffer containing 0.025 M Tris-HCl (pH 6.8), 2% SDS, 5 mM 2-mercaptoethanol and 10% glycerol and heated for 10 min at 100 °C. Electrophoresis was carried out at 50 V until the sample had passed through the spacer gel, after which 100 V was applied. Gels were stained with Coomassie Brilliant Blue R250 (0.2 g in 100 ml 7% acetic acid in methanol–water 1:1 v/v). After destaining in the solvent, gels were treated with DMSO–PPO, dried and placed in contact with RP Royal X-Omat X-ray film at –70 °C for 2.5 months¹⁴.

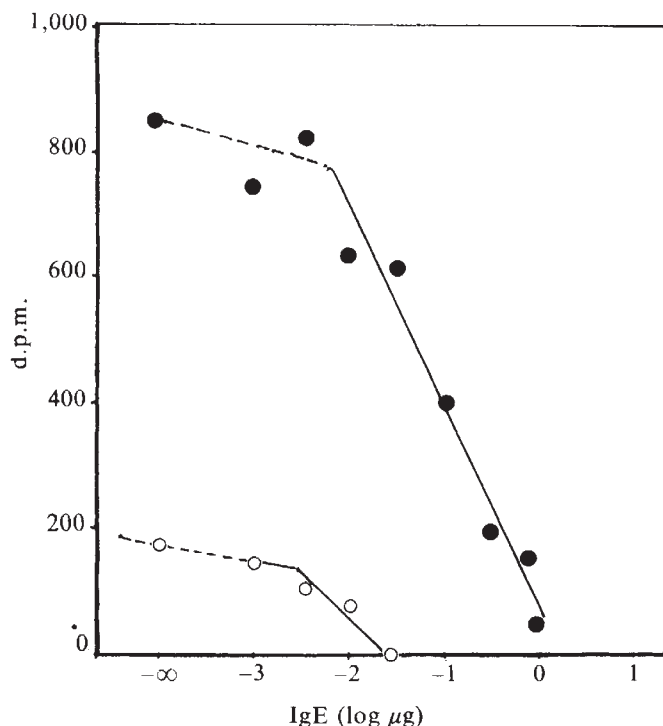


Fig. 3 Dilution experiment by coincubation with purified rat IgE. Before addition of goat anti-rat IgE as described in Fig. 1, the indicated amounts of purified rat IgE (ref. 13) were mixed with the samples. Further procedures were as in Fig. 1 and control values were subtracted. ●, Twelve cells injected per egg, ○, one cell injected per egg.

Electrophoretic analyses of the translation products with myeloma and endogenous RNA are shown in Fig. 2. Most of the radioactivity is found in those parts of the gel where the IgE marker bands are found, whereas in the control experiment, radioactivity was almost exclusively in the buffer front. Further proof of specific translation is given in Fig. 3, which shows that the precipitation of radioactivity with anti-rat IgE is diminished by coincubation with unlabelled purified rat IgE. The results of a similar experiment with the translation products after single cell injection are more variable, but they show the same tendency.

We conclude, therefore, that the *Xenopus* egg translation system makes possible study of the activity of a small number of cells, which can be selected under the microscope by micromanipulation and, as a result, can be very well defined. The minimum number of cells to be used will depend on the amount of messenger per cell, but in our experiments, we could even go as far as a single cell.

Another and, in our view, a very important advantage of our technique, is the fact that no extraction procedures are involved. Part of every RNA extraction is deproteinisation. As a result, masked messengers will contribute their products, whereas they would be inactive *in vivo*. Our method better preserves the native structure of the RNA and, therefore, gives better insight into the actual translational processes in the cell.

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***In vitro* synthesis and characterisation of full- and half-genome length complementary DNA from avian oncoviruses**

RNA TUMOUR viruses carry an RNA-dependent DNA polymerase which can transcribe the viral RNA genome into single-stranded and double-stranded DNA in detergent-disrupted virions^{1,2}. For several years since the discovery of this enzyme, only short DNA fragments of about 100-200 nucleotides long could be produced³. Recently, conditions have been described for the synthesis of possibly full-genome length complementary DNA (cDNA)⁴⁻⁶. The *in vitro* conditions described, however, were not well characterised and did not seem to be very efficient. We report here an efficient synthesis *in vitro* of half- and possibly full-genome length cDNA in avian oncoviruses. These two discrete species of cDNA were synthesised in large quantities. Furthermore, some of the half-genome length cDNA were found to form single-stranded circular molecules with a distinct secondary structure.

The Schmidt-Ruppin strain of Rous sarcoma virus (RSV) of subgroup D (SR-D) was used throughout the experiments. The similar procedures have also been successfully applied to several other strains of avian and murine oncoviruses. Growth of avian oncoviruses in secondary chicken embryo fibroblasts followed the published procedures⁷. The culture media were collected every 12 h. The viruses were purified according to the published methods⁸, and were used immediately for DNA synthesis as described in Fig. 1. We have previously observed that the optimum concentration of Triton X-100 used in the DNA synthesis varied for every virus preparation. It was therefore determined individually for every virus preparation. We also found that the minimum concentration of the viral proteins for efficient synthesis of large cDNA (molecular weight $> 1.5 \times 10^6$) was 1 mg ml^{-1} . At lower concentration, the efficiency of DNA synthesis remained the same, but the molecular weight of DNA products were usually less than 1×10^6 . In contrast to the published report⁹, we found that sodium pyrophosphate inhibited rather than enhanced DNA synthesis (data not shown).

The DNA products synthesised *in vitro* were analysed by using an alkaline sucrose gradient. As can be seen in Fig. 1a, 10-20% of the DNA synthesised had a sedimentation velocity greater than or equal to that of the denatured SV40 DNA marker (molecular weight 1.65×10^6). There were two distinct peaks of DNA products. The one with higher sedimentation velocity (peak I) comigrated with ³²P-35S viral RNA when electrophoresed in a formamide-acrylamide gel (Fig. 1b). This DNA was also shown by electron microscopy to be single-stranded linear DNA

molecules of 9–10 kilobases. About 90% of these DNA molecules could be digested with nuclease S1. After hybridisation with the viral 35S RNA, however, they became completely resistant to nuclease S1 (Table 1). Thus, this DNA peak represented single-stranded cDNA molecules, most of which were near full-genome length. They represented about 5–15% of the total cDNA made. At least 1 μ g cDNA of this species could be synthesised from 5 mg of the purified viruses.

The peak II DNA from the alkaline sucrose gradient had a sedimentation rate roughly equivalent to that of denatured SV40 DNA. This peak consisted of linear DNA molecules of 5–6 kilobases by electron microscopy. As with the full-genome-length cDNA, about 90% of the peak II DNA was sensitive to nuclease S1; after hybridisation with the viral 35S RNA, however, it became completely resistant (Table 1). Thus, they also represented complementary DNA sequences. In three independently prepared lots of cDNA from SR-D, circular single-stranded DNA molecules were observed besides linear DNA molecules (Fig. 2a and b). These circular molecules were very homogenous in size and the contour length of the circle was 5.63 ± 0.38 kilobases, which corresponded to a molecular weight of about 1.8×10^6 . A secondary structure resembling 'rabbit-ears' of distinct length (1.34 ± 0.23 kilobases if traced as completely duplex) and shape were also present in all of the circular DNA molecules (Fig. 2a and b). These circular molecules represented approximately 2% by number of the cDNA from peak II. We believe that these circular molecules were the product of the viral reverse transcription and not cellular contaminants, because no such molecules could be detected in the nucleic acids from the same SR-D virus preparation which had been subjected to the identical purification procedures, including RNase digestion and alkaline sucrose gradient sedimentation. In high formamide hybridisation conditions, these half-genome-size circular DNA could hybridise to the 35S viral RNA genome (for example, Fig. 2c). Unfortunately, such hybrids could only be detected at a very low frequency so that we could only tentatively map the hybrid region at the 5' half of the viral genome, starting at 3.5 kilobases from the 3' end of the genome. The

Table 1 Resistance of the *in vitro* synthesised DNA to nuclease S1

	After hybridisation without RNA	After hybridisation with RNA
Peak I	12%	100%
Peak II	12%	100%
Peak III	39%	85%
Peak IV	29%	79%

1,000 c.p.m. (specific activity $50,000 \text{ c.p.m. } \mu\text{g}^{-1}$) of the *in vitro* synthesised ^3H -cDNA separated as in Fig. 1a with or without 1 μ g of the 70S viral RNA was used in each experiment. Hybridisation was carried out in 100 μ l of the solution containing 0.9 M NaCl, 0.09 M sodium acetate, 0.05% sodium dodecyl sulphate (SDS) and 1 μ g yeast RNA. The hybridisation mixture was heated at 120°C for 1 min, chilled immediately and then incubated at 67°C for 30 min. Afterwards, the hybridisation mixture was cooled gradually over a 12-h period. The nucleic acids were precipitated with 3 volumes of ethanol and then dissolved in 100 μ l of a solution containing 0.03 M sodium acetate buffer, pH 4.5, 0.0018 M ZnCl_2 , 0.3 M NaCl and 200 units of nuclease S1 (Miles Laboratories)²⁰. The reaction mixture was incubated at 37°C for 2 h and the percentage of the ^3H -cDNA resistant to nuclease S1 was determined from trichloroacetic acid (TCA)—precipitable counts before and after nuclease S1 digestion.

difficulty in observing this kind of hybrid probably stemmed from the fact that, in the hybridisation conditions used, RNA·DNA hybrid is more stable than DNA·DNA duplex region which is responsible for the circle formation¹⁰. Therefore, most of the hybrids formed between the 35S RNA and the circular half-length cDNA would have become linear.

Thus we have made *in vitro* in high efficiency full- and half-genome-length cDNA from avian oncoviruses. It is interesting to note that the DNA molecules of molecular weight greater than 1.5×10^6 contain only complementary sequences. The shorter DNA fragments, on the contrary, contain both (+) and (−) strands as evidenced by their partial sensitivity to nuclease S1 after hybridisation with the viral RNA (Table 1). The full-length cDNA (peak I) was probably derived from reverse transcription initiated at the primer site to the 5' end of the virion RNA¹¹. The transcription complex may then have jumped to the 3' end of the same or adjacent genome and continued synthesis until

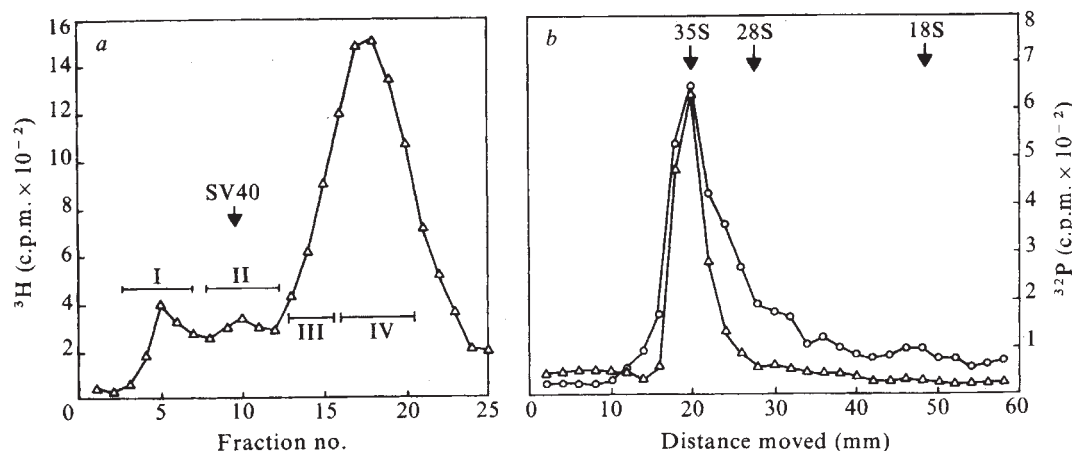


Fig. 1 Characterisation of the ^3H -DNA products synthesised *in vitro*. The viruses purified from sucrose gradient⁸ were concentrated by sedimenting on to a 65% sucrose- D_2O cushion containing 0.01 M Tris-HCl, pH 7.4, 0.1 M NaCl and 1 mM EDTA before being used for DNA transcription. The endogenous reverse transcription was carried out in 4–8 ml of the reaction mixture containing 0.1 M Tris HCl, pH 8.0, 30 mM DTT, 3 mM magnesium acetate, 1 mM each of dATP, dCTP, and dGTP, 0.5 mM ^3H -TTP (200 mCi mmol⁻¹, New England Nuclear), 1 mg ml⁻¹ of the purified viruses, and 0.02–0.03% of Triton X-100. The optimum Triton concentration was determined beforehand. The reaction mixture was incubated at 40°C for 18 h. The DNA transcription was stopped by addition of EDTA to a final concentration of 12 mM. The nucleic acids were extracted with phenol three times and then treated with RNase A ($50 \mu\text{g ml}^{-1}$) at 37°C for 1 h. The ^3H -DNA products were then further phenolised twice before being precipitated with 3 volumes of ethanol. *a*, Alkaline sucrose gradient sedimentation of the total DNA products. The ^3H -DNA was pelleted by sedimentation at $20,000g$ for 15 min and then dissolved in 0.3 ml of a solution containing 0.33 M NaOH, 0.5 M NaCl and 0.02 M EDTA. The sedimentation was carried out in a 5–20% sucrose gradient containing the same solution at $40,000 \text{ rpm}$ for 11 h at 20°C in an SW41 rotor; 0.5-ml fractions were collected, and an aliquot of each fraction was counted. *b*, Fractions 3–6 in *a* were pooled, neutralised with 1 M acetic acid and then precipitated with 3 volumes of ethanol. The precipitate was dissolved in 40 μ l of phosphate-buffered formamide and electrophoresed in 2.5% formamide-polyacrylamide gel at 80 V for 18 h as described previously¹⁸. The ^{32}P -35S RNA of SR-D was included as a marker. 28S and 18S RNAs were run in a parallel gel. Δ , ^3H c.p.m.; $\circ$, ^{32}P c.p.m.

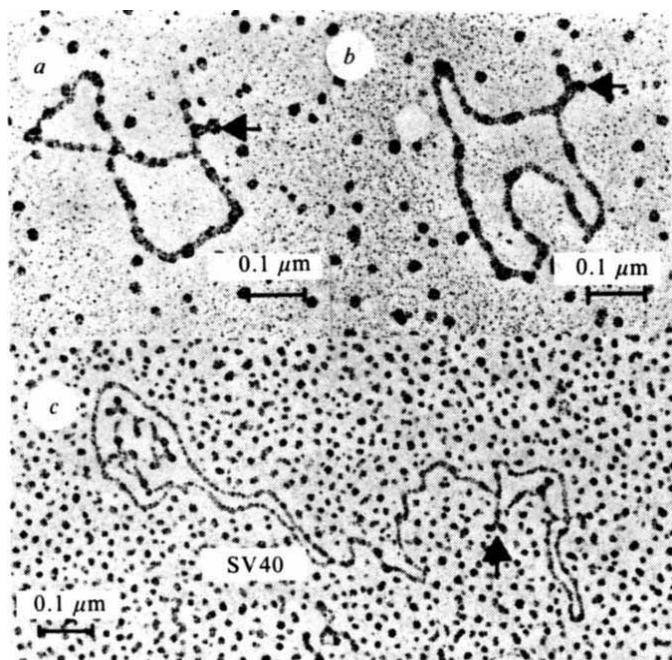


Fig. 2 Electron micrographs of the cDNA synthesised *in vitro*. *a* and *b*, Fractions 8–12 in Fig. 1*a* were pooled and pelleted with ethanol. The DNA was dissolved in 50 μ l H_2O . Aliquots of the DNA were spread from 55% formamide, 0.06 M Tris-HCl (pH 8.5) and 0.006 M EDTA on to a hypophase containing 18% formamide, 0.01 M Tris HCl, pH 8.5, and 0.001 M EDTA. The arrows denote 'rabbit-ear'-like structures. *c*, Heteroduplex between circular cDNA and 35S RNA from SR-D. cDNA 20 μ g ml⁻¹ was hybridised to 35S RNA 10 μ g ml⁻¹ in a solution containing 80% formamide, 0.4 M NaCl, 0.01 M pipes (pH 6.40) at 46 °C for 30 min. Aliquots of the hybrids were then hybridised to SV40-BrdU (that is, SV40 DNA on to which short poly(BrdU) tails have been added by terminal transferase¹⁹ at room temperature for 20 min in the presence of 0.25 M Tris and 0.025 M EDTA, pH 7.50). The final product was spread with the urea + formamide method described by Kung *et al.*¹⁵.

it reached near the 5' end again¹². Analysis of the DNA fragments of this full-length DNA cleaved by restriction endonucleases supported this interpretation (J. Taylor, A. S. Hsu and M.M.C.L., unpublished). It is not clear, however, where the DNA synthesis terminates. The half-length cDNA might be the result of early termination of transcription. Alternatively, it might have initiated from the same primer near the 5' end and then jumped to the middle of the RNA template. The latter interpretation is consistent with the observations that the early DNA products of reverse transcription contained sequences complementary to those in the middle of the 35S RNA^{13,14}. Preliminary heteroduplex studies suggested that at least part of the half-length cDNA was derived by the latter mechanism. Since the half-length circular cDNA molecules were tentatively mapped at the 5' half of the viral genome, they, too, were probably derived by the latter mechanism.

The formation of the 'rabbit-ear'-like structure in SR-D half-size circular DNA is also curious. This feature is similar to the dimer linkage structure of the RD-114 RNA genome in terms of size and shape¹⁵. We do not know whether it is all double-stranded in this region. Moderate denaturing conditions (55% formamide) did not dissociate this structure. Two possible mechanisms could account for the formation of such structure: (1) If these DNA molecules were the faithful transcripts of the viral genome it would suggest that there are inverted complementary sequences within the viral genome, spaced about 5.6 kilobases. A similar arrangement of genetic sequences has been observed in the genome of herpes simplex virus¹⁶. This arrangement, however, is unlikely since we did not observe such structures in full-length cDNA or 35S viral RNA. (2) If these half-size cDNA molecules represented the products

of aberrant DNA synthesis, for example, a hairpin synthesis at the termination site, then there might be homologous repeated sequences at both the initiation and termination sites for some linear half-size cDNA, which can then form a 'rabbit-ear'-like hybrid region. It has been demonstrated that small double-stranded circular viral DNA of molecular weight 3.4×10^6 could be found in the cells infected with Rous sarcoma viruses (ref. 17 and H. J. Kung, personal communication). The function and fate of these half-size circular viral DNA are unclear. They may be related to the half-size single-stranded circular cDNA of molecular weight 1.8×10^6 we observed in the endogenous reverse transcription *in vitro*.

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Virus-like particles pathogenic to salivary glands of the tick *Boophilus microplus*

TICK-BORNE viruses are generally transmitted into the host's vascular system in infected salivary secretions¹, and it was to be expected that virus-like particles (VLPs) would be observed during an ultrastructural study of the salivary gland of the cattle tick *Boophilus microplus*. But it is surprising that some of these particles, which are described here, seem to damage the cytoplasm of infected cells, and in extreme cases even to affect the secretory competence of the salivary glands. In view of the economic and medical importance of these arthropods, viruses pathogenic to ticks could have a potential as biological control agents.

Adult female ticks were reared in the laboratory with stalled calves as hosts. VLPs were observed in three different generations collected for 6 months. Salivary glands were prepared as described elsewhere².

Secretory cells within the salivary glands of infected ticks contained large inclusions of regulatory aligned VLPs (Fig. 1). Similar particles were not seen in either the synganglion or peripheral nerves from the same ticks. Less heavily infected cells were evident in which individual spherical particles were distributed randomly throughout the cytoplasm. The particles had a diameter of 42–45 nm and consisted of an electron-dense inner core (28–31 nm diameter) surrounded by a less dense outer rim. Some cells from infected tissues were characterised by cytoplasmic

areas which appeared light in intensity when compared with neighbouring regions. These 'plaque' regions were devoid of cytoplasmic organelles and contained both individual VLPs and groups of paracrystalline aggregates (Fig. 2).

In densely infected cells the particles were not observed as paracrystalline arrays but as individual particles which still had the electron-lucent outer rim and the electron-dense inner core (Fig. 3). Occasionally long crystalline filaments were observed in heavily infected cells; these filaments had an electron-dense outer layer and an

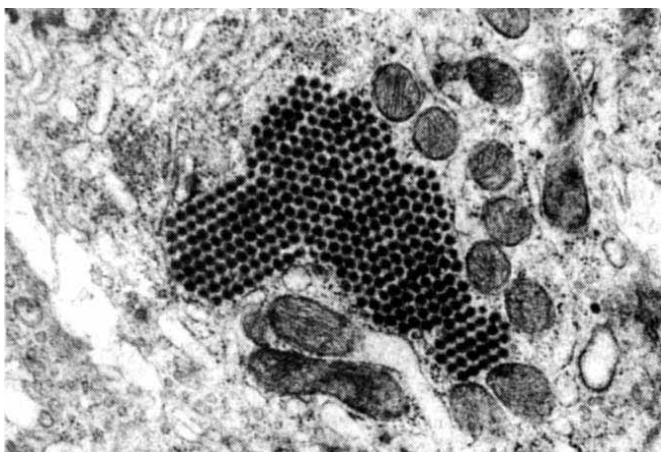


Fig. 1 Group of VLPs in granule-secreting cell from a type II acinus (50,555).

electron-lucent inner core—the reverse arrangement to that seen in the VLPs. It is not yet clear whether the filaments represent a different form of the particle (for example, a self-assembling aggregation of cell synthesised viral protein in excess of that required for virus-particle assembly) or if they are indicative of a different VLP. Similar filaments have been observed in close association with Colorado tick fever virus in cultured hamster kidney cells and in mouse brain cells³.

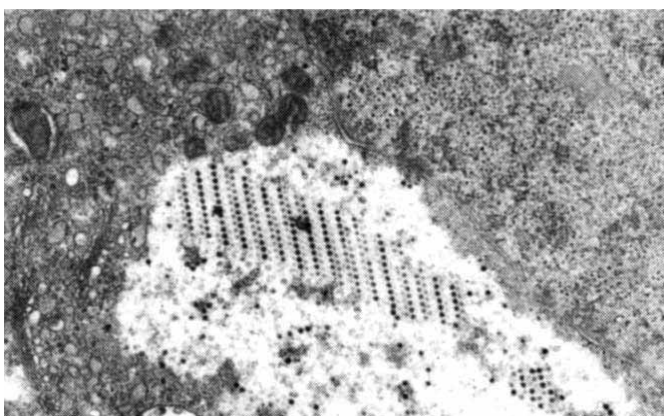


Fig. 2 Plaque region in the cytoplasm of an infected tick (33,000).

Densely infected cells were only seen in salivary glands from abnormally engorged ('plasma') ticks, that is ticks containing large volumes of haemolymph (100–150 μ l, compared with the 'normal' volume of 10 μ l)⁴. These 'plasma' ticks have been assumed to have fed on body fluids other than blood. Such an explanation would account for the low haemocrit value of the gut contents⁵ but not for the large haemolymph volumes. It is possible that the dense infections of VLPs observed in the salivary glands of 'plasma' engorged

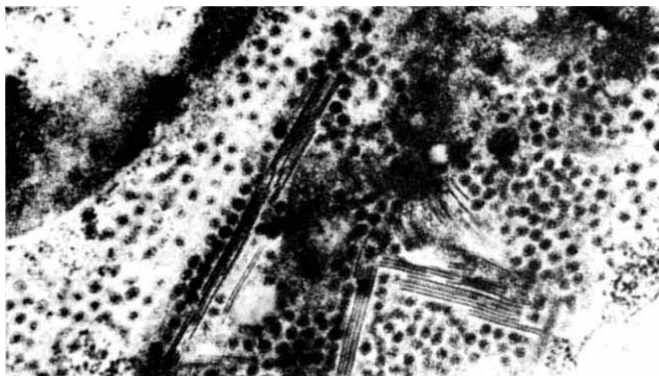


Fig. 3 Two distinct types of VLP in a granule-secreting cell from a salivary gland of a 'plasma' engorged tick.

ticks reduce the secretory potential of the glands resulting in the accumulation of fluid in the haemocoel.

The presence of the particles described here has been established purely on morphological grounds. Studies directed at the confirmation of the viral nature of the particles and their pathogenic effects are continuing. Some viruses are known to be pathogenic to mites⁶ but so far none has been described in ticks.

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Errata

In the letter by R. R. Kay, D. Garrod & R. Tilly, *Nature* 271, 58–60, the title should read 'Requirements for cell differentiation in *Dictyostelium discoideum*'.

In the letter 'Sequences and efficiencies of proposed mRNA terminators' by J. E. McMahon & I. Tinoco, Jr, *Nature* 271, 275, in paragraph 2 line 1, for give read given. In the text figure representing the sequence for two base pairs the dots should be centred between G–C and C–G.

Nature Index and Binders

The complete **Index** for 1976 is available, price £2.50 (UK), US\$5.00 (Rest of World). Copies of the 1975 index are still on sale, price £2.25 (UK), US\$5.00 (Rest of World).

Binders for the journal are also available at £8.00 (UK), US\$16.00 (Rest of World) for three: a year of *Nature* fits into three binders.

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reviews

Territoriality and adaptation

Jared M. Diamond

Evolutionary Ecology. Edited by Bernard Stonehouse and Christopher Perrins. Pp. vi+310. (Macmillan: London, 1977.) £12.95.

THE late David Lack was a distinguished ornithologist noted for his pioneering evolutionary studies of ecological problems, such as ecological segregation among species, population regulation, territoriality, and reproductive rates. The present volume (not to be confused with the textbook of the same title, by Eric Pianka) consists of a collection of 21 chapters on topics related to Lack's interests, by former students, colleagues, scientific adversaries, and workers whom he influenced at a distance.

The chapters are grouped into four sections. The first five chapters discuss population regulation and territoriality, as illuminated by red grouse (A. Watson), wild rabbits (J. A. Gibb), and great tit (J. R. Krebs), or by controversies that pitted two of the authors (V. C. Wynne-Edwards and D. Chitty) against Lack. Next come four chapters that explore feeding adaptations and ecological segregation in Galapagos seabirds (M. P. Harris), marine Pelicaniformes (J. B. Nelson), seed-eating rodents (M. L. Rosenzweig), and common terns (I. C. T. Nisbet).

The longest section, on breeding adaptations and reproductive rates, consists of eight chapters discussing: arctic geese (I. Newton); great tit (H. N. Kluyver, J. H. van Balen, and A. J. Cavé); cooperative breeding among birds in general (C. H. Fry) and Argentine blackbirds in particular (G. H., C. E., and K. J. Orians); and clutch size in birds (D. F. Owen, C. M. Perrins and R. E. Ricklefs) and in the plant family Compositae (D. A. Levin and B. L. Turner).

The book concludes with four miscellaneous chapters, grouped under the heading of evolutionary adaptations, on systematics of echolocating swiftlets (Lord Medway and J. D. Pye), male group displays in *Chiroxiphia* manakins (D. W. Snow), animal communication systems (A. Zahavi), and big-bang reproduction in yuccas and agaves (W. M. and M. V. Schaffer).

To produce a memorial volume written by 21 different authors or sets

of authors, spanning a wide area of population biology, short enough to sell at a reasonable price, and of high scientific interest is at best a difficult task. The conflicting requirements of these goals weaken the book. Chapters average a frustratingly brief 12 pages in length. This prevents each chapter from giving a major overview of a field or exploring one problem in satisfying detail. The diversity of the content, and the paucity of cross-references between chapters, prevent the volume from developing a 'critical mass' on any single subject except nesting biology. Chapters vary considerably in level of insight. Some good chapters, such as those by Krebs and by Medway and Pye, seem to be so unrelated to the rest of the book that they would have been more effective if they had been published separately as journal articles.

The brevity of chapters is especially unfortunate because population regulation and reproductive rates are complexly determined by multiple factors, the relative importance of which varies with circumstances and with species. In the past, these subjects have often been approached in single-factor, single-species treatments, leading to running controversies that retarded progress. The sections on these two subjects would each have profited from a lengthy chapter synthesising the major factors into an overall scheme, reviewing each factor in detail, and giving examples of species or circumstances where each is important, to place the following chapters in perspective. Overall, I feel that this volume would have been more effective if it had focussed on a narrower span of population biology and consisted of fewer, longer chapters.

These remarks about the book as a whole do not detract from the interest of some chapters taken individually. Three examples must suffice. Watson reviews experiments and observations on the role of territoriality in Scottish red grouse. Between August and November of each year, males establish territories, and females pair with territorial males. The territory provides its owners with exclusive access to food in winter and spring, when food is scarcest and females are forming eggs. Most non-territorial males and unmated

females die of predation or of food scarcity by May. If territorial birds are shot, non-territorial individuals take over the territory, survive well, and breed. Brood size is determined in part by food supply mediated by egg quality formed by females: artificially fertilising the heather results in large broods. Implanting androgen hormone pellets in males makes them more aggressive, and results in their acquiring territories if initially non-territorial or enlarging territories if already territorial.

Newton discusses the extreme set of breeding adaptations that Arctic geese have evolved in response to the short summer. The geese arrive from their southern wintering grounds mated, the females fertilised and ready to lay within a week. Until eggs hatch, the adults survive mainly on fat and protein reserves laid down at wintering and migration areas, and they lose 17–50% of their initial weight. Number of eggs per clutch is greater for early-season clutches or in years with early snow melt. Breeding success varies enormously among years, so that the contribution of young birds to the autumn population may be as low as 1% or over 50%.

Zahavi illustrates the costs incurred by animal communication systems to achieve reliability and minimise cheating. Baby birds beg food from parents by displays that attract the parents' attention but could also attract predators. This risk 'blackmails' the parent into bringing food but inhibits fed nestlings from begging without cause and thereby diverting parents from unfed siblings. Arabian babblers live in groups which jointly defend a territory but of which only one, dominant pair breeds. The dominance hierarchy is maintained not by constant fighting but by a superficially altruistic display: dominant birds offer food to subordinates. That this display represents a dominance manoeuvre rather than altruism becomes clear when a subordinate individual offers food to a dominant one: this constitutes an act of rebellion and immediately provokes a fight. □

Jared M. Diamond is Professor of Physiology at the University of California Medical Center, Los Angeles, California.

Catastrophe theory

Catastrophe Theory: Selected Papers 1972-1977. By E. C. Zeeman. Pp. 675. (Addison-Wesley: Reading, Massachusetts, 1977.) Hardback \$26.50; paperback \$14.50.

THE strange union of mathematics, philosophy and methodology that has come to be called catastrophe theory sprang entirely from the totally original mind of René Thom. But its widespread popularisation, and its detailed application to the most diverse subjects, is largely the work of Christopher Zeeman. Their writings provide a perfect example of the contrast between the Gallic and Anglo-Saxon intellectual styles—while Thom is grandiose, poetic, elliptic, allusive, impatient with practical detail, sceptical about the possibility that his theory can be put to experimental test, Zeeman is pragmatic, painstaking, pedagogic, tirelessly fashioning ingenious falsifiable models of particular experimental situations, confident that catastrophe theory will form part of the mainstream of applied mathematics.

Thom's great book *Structural Stability and Morphogenesis* has been available since 1972, but Zeeman's most important work has appeared in conference proceedings or journals that are often difficult to obtain. Therefore this publication of a collection of twenty-two of his papers is a very welcome event—all the more so because Zeeman's work has recently come under strong critical attack. Now readers can study the original material and arrive at an informed opinion on the subject.

The mathematics gets steadily more difficult as the book progresses, the culmination being a proof of Thom's theorem (on the classification of singularities of gradient maps—the 'elementary catastrophes') which will be heavy going even for pure mathematicians. The bulk of the book, however, is devoted to applications of the theorem, and it is fortunate that it is possible to understand what it states, and to apply it, without knowing anything of the proof. In restricting himself to applications of the classification theorem (particularly the cusp catastrophe), Zeeman differs from Thom himself, whose speculations often invoke 'generalised catastrophes' the mathematics of which hardly exist as yet.

Space prevents my giving more than a brief indication of the range of Zeeman's applications. At the 'softest' level, they consist of dramatic pictorial encapsulation of the sense of proverbs using the cusp catastrophe; my favourite is "more haste less speed", where haste and skill are conflicting influences

(control parameters) on speed (behaviour variable). Then there are highly developed but still qualitative applications to biology, sociology and psychology. One example is the analysis of the development of the nervous disorder *anorexia nervosa* in terms of the cusp, the hysteresis of which, increasing with the 'splitting factor', models sufferers' sudden alternations between fasting and gorging; a clever invocation of the extra stable sheet of the butterfly catastrophe enables Zeeman to describe the way in which trance therapy can effect a seemingly miraculous cure. In two models (for the onset of prison riots and the impairment of driving skill by alcohol), quantitative data are presented; these are supposed to fit cusp catastrophes, but the points scatter so much that I suspect most readers will share my reaction of being impressed by Zeeman's ingenuity while remaining unconvinced by the evidence.

The 'hardest' applications are in physics. His use of the cusp to describe phase transitions is equivalent to Van der Waals' theory which is well known to fail at the critical point. Of this he

writes: "since density is an averaging device, the shape of the equilibrium surface very near the critical point is slightly distorted". By implication, this dismisses a flourishing area of theoretical physics aimed at understanding the deep reasons for this failure, and such naiveté is likely to cause physicists to think that catastrophe theory can make only trivial contributions in their subject; and that would be a pity. Two of the best chapters in this book, however, also concern classical physics. The first is the elegant and powerful summary of elastic buckling theory. The second is the masterly analysis of the stability of ships, where Zeeman blends the qualitative and the quantitative in an inimitable blend of orthodox naval architecture, topology, group theory and seamen's lore.

My opinion is that for readers interested in applications of catastrophe theory this is the best currently available introduction to the subject.

Michael Berry

Michael Berry is Reader in Physics at the University of Bristol, UK.

Ion-containing polymers

Ion-Containing Polymers: Physical Properties and Structure. By A. Eisenberg and M. King. Pp. 287. (Academic: New York and London, 1977.) \$27.50; £19.55.

THIS second volume in Academic's new series on Polymer Physics presents the first unified treatment of the physical properties and structure of *Ion-Containing Polymers*, which, according to the authors' definition, embraces polymer systems that contain ions and those to which ions can be attached. As one would expect, the book has been thoughtfully planned, no mean feat in a rapidly growing field. It is prefaced by a useful list of symbols; each chapter has a good bibliography; and there are separate author and subject indexes. The format is attractive and the print clear; the vast majority of figures are also excellent but good eyesight is needed to discern detail in a few; CGS units are used.

An introductory chapter describes current knowledge and gives a useful general classification scheme for ion-containing polymers subdivided into two main groups—polymers of low ion content, including Nafion-type systems and ionomers, and polyelectrolytes.

Chapter 2 deals with supra-molecular structure and glass transitions. Theories of and experimental

evidence for multiplet and cluster formation are treated in some detail, although not to mathematical excess. Clustering is seen to be akin to micro-phase separation and to result in many characteristics normally associated with crystalline systems.

Chapters 3 and 4 on the viscoelastic properties of homopolymers and copolymers respectively deal broadly with polyphosphates and silicates; low molecular weight salts in polar polymers and charge transfer complexes; polyelectrolytes; non-crystalline copolymers of high T_g (including perfluorinated ionics, rubber-based ionomers and crystalline copolymers mostly based on ethylene); and polyelectrolyte complexes.

Amongst the properties detailed are stress relaxation, creep compliance, modulus, mechanical loss, specific viscosity, zero shear viscosity, dynamic viscosity, normal stress coefficient and dielectric constant.

The final chapter (5), on configuration-dependant properties, discusses polyelectrolyte dimensions in solution, rubber elasticity, and dilute solution viscosity.

A valuable state-of-the-science book for the research worker in, or wishing to enter, the field of ion-containing polymers, as well as for the professional academic or industrialist.

P. L. Clegg

P. L. Clegg is Head of the Polymer Science Division of the Research Department at ICI Plastics Division, UK.

Notable Victorian naturalist

William Carmichael M'Intosh. By A. E. Gunther (Scottish Academic Press: Edinburgh, 1977.) £5.

THE way of the aspiring academic zoologist in the last century, not least in Scotland, was difficult. It usually involved initial training in medicine. Forbes, Wyville Thompson and Darwin failed to stay the course at Edinburgh. Perhaps W. G. M'Intosh was less fortunate, because graduation qualified him for 22 years' service in lunatic asylums before, in 1882, being appointed to the singularly designated chair of Civil and Natural History at St Andrews. There, however, he remained until 1917, retiring in his 80th year with fourteen years of activity still ahead.

Lifelong interest in marine biology began in childhood rambles on shores around St Andrews, from which his parents and sisters sent parcels of specimens during his years at the Perth District Asylum. He was a naturalist interested in comparative anatomy—highly conventional, holding Darwin in high regard as a naturalist but never really accepting evolution. His name will always be associated with the volumes of his Ray Society Monograph on British Annelids. Taxonomy was a subject to be treated with respect, and frivolities such as the naming, even by Ray Lankester, of a local sipunculid as *Golfingia macintoshi* were to be deplored.

He was involved in early activities

of the Scottish Fishery Board and in the controversies concerning the effects of intensified fishing. He maintained in his *Resources of the Sea* that nothing man did could materially diminish productivity. Pleas for a marine laboratory were answered by a generous donor from Sussex and the Gatty Marine Laboratory was opened in 1896. It was a minor tragedy when the small government grant was stopped and a greater one when his successor, formerly "my junior colleague at Dundee", D'Arcy Thompson, closed it. Already he had offended M'Intosh by participation in the much criticised activities of the International Council for the Exploration of the Sea. Indeed, apart from longevity, no two zoologists could have had less in common.

M'Intosh was a bachelor cared for by his sisters, the youngest of whom, responsible for early illustrations of his annelids, married Albert Gunther, Keeper of Zoology in the British Museum. It is this association that, following his *Century of Zoology*, has led A. E. Gunther to write this welcome account of his great uncle. Based on an unpublished autobiography and a wealth of documented information, it perpetuates the memory of a notable Victorian naturalist and a worthy fighter in what he believed to be right causes. With what happiness would he not view the present flourishing state of the Gatty Laboratory.

C. M. Yonge

Sir Maurice Yonge is Honorary Fellow in Zoology at the University of Edinburgh, Scotland.

Proteins in brain function

Protein Metabolism of the Brain. By A. V. Palladin, Ya. V. Belik and N. M. Polyakova. Pp. 335 (Plenum: New York, 1977.) \$42.

THERE is an active school of Soviet neuroscientists and Professor A. V. Palladin, who died in 1972 at the age of 87, was one of the pioneers. Palladin was particularly interested in the biochemical basis of brain function and this book reflects his interest in the role of proteins in the structure and organisation of the brain.

The book contains a description, usually in chronological sequence, of research into brain protein metabolism, with special reference to the contributions of Soviet scientists. The book was first published in 1972, in its original Russian, the English translation being completed in 1977. Because of this un-

fortunate lapse in time, it has become dated with the latest references being from 1970. Since then, there has been considerable progress in the effects of stress, hormones and drugs on brain protein metabolism. Moreover, our ideas on reutilisation of amino acids and turnover in the brain have undergone revision.

Not surprisingly, much of the basic information described under various subheadings—for example, "Brain Protein Metabolism in Ontogeny" or "Catabolism of Nerve Tissue Proteins"—can be found in more recent reviews dealing separately with these subjects. Nevertheless, the book does have the advantage of bringing together in one volume, the older literature on the varied aspects of brain protein metabolism, together with relevant Soviet literature. Even by today's standards, however, this is an expensive book.

Louis Lim

Louis Lim is Senior Lecturer in Neurochemistry at the Institute of Neurology, London, UK.

Eel biology

The Eel: Biology and Management of Anguillid Eels. By F. W. Tesch. Pp. 434. (Chapman and Hall: London, 1977.) £18.

WHEN I reviewed for these columns the original 1973 German version of this book, I expressed the wish to see it in English. This has now been granted, and Miss Jennifer Greenwood has given us a smooth and impeccable translation.

The sections concerned with endocrinology and sex determination have been brought up to date by Dr Ian W. Henderson. It is a pity that they were not expanded more. For example, two paragraphs on the interrenal organ (adrenocortical analogue) bulldoze over 19 references. Readers would welcome something more satisfying than the enumeration of papers, given that these topics are the growing points of current research on the eel. In fact, 50 out of 98 or so new (post-1972) references given are in the field of physiology. They cover also the feat of inducing sexual maturation of eels in the laboratory.

By contrast, it would seem from the references, that only Dr Tesch himself is currently engaged in the analysis of the eel's migratory behaviour.

The parts on fishery aspects remain the most detailed of the book. The continued interest in the economic value of the eel is shown by the fact that 26 post-1972 papers cited deal with fisheries and fish culture. This is well deserved by an animal that converts into protein for human consumption the insect larvae, worms and snails of rivers and lakes, that is, materials that could not be put to use in other ways. It is interesting to read of the persistent eel re-stocking operations in Eastern Europe and the commercial eel cultures in the Far East.

There have been minor adjustments in other parts of the book too; but no Table nor Figure has been replaced or added on the way. Even the prices of eels are still shown in Table 41 at their 1963 level!

The eel still presents fascinating and instructive problems for investigation. Its physiological versatility, its ambiguous sex determination, the intriguing migration patterns and separation of species, are only some of these. This good English version covering over 1,000 references on the subject, will be of great value to a much wider circle of biologists.

E. M. Pantelouris

E. M. Pantelouris is Reader in Genetics at the University of Strathclyde, UK.

obituary

Maurice Ingram

DR MAURICE INGRAM, former Director of the Agricultural Research Council Meat Research Institute at Langford and Professor of Applied Microbiology at the University of Bristol from 1968 to 1973, died suddenly at his home in Churchill, Avon, on Tuesday 15 November 1977.

Dr Ingram, who was born in Bradford in 1912, was educated at Bradford Grammar School and had a distinguished undergraduate career at Queens' College, Cambridge. After an early interest in botany he became increasingly concerned with food microbiology. His Ph.D. dissertation on *The Effect of Salts in Bacterial Respiration* was presented after joining the Low Temperature Station for Research in Biochemistry and Biophysics where he became Head of the Microbiology Department in 1951.

His early research on bacterial growth and metabolism led to studies of the involvement of pre- and post-slaughter stress factors in the quality and stability of cured products. This work became particularly significant during the second world war when he was engaged in problems of transporting meat and with its salvage after enemy action. In addition he directed research that led to the development of the special rations issued to Allied invading armies and to underground organisations in Europe. He later received the Haakon VII Liberty Medal from the Norwegian Government for his contribution to the partisan attack which destroyed the heavy water plant at Rjukan.

From 1947-49 he studied the bacteriology of whalemeat in an effort to utilise this wasted source of protein. His work on the preservation of concentrated orange juice, which involved travelling to Israel, Italy and Spain, led to the elimination of serious losses. Subsequently he led a team investigating the preservation of foods by ionising radiation. His earlier work again became important in further studies of curing, particularly with reference to food safety and the control of *Clostridium botulinum* by curing salts. He was also active in developing microbial specifications for foods.

In 1963 Dr Ingram was appointed the first Director of the projected Meat Research Institute and as he also became Director of the Low Temperature Station in 1965 he was involved in

the planning and recruitment for the two Institutes which replaced it, the Food Research Institute at Norwich and the Meat Research Institute at Langford. The latter was opened by H.M. the Queen in 1968 and Dr Ingram remained Director until 1973; an honorary degree of Doctor of Veterinary Medicine being conferred on him by the Ludwig-Maximilians University of Munich that year followed by the CBE in 1974.

He retired earlier than necessary to avoid ever-increasing administration which he did not enjoy. Retirement meant that he was able to devote himself more fully to science, and his international reputation ensured that he continued to travel widely, both in a personal capacity and as a consultant for the World Health Organisation. In July 1977 he received the Polish Society of Microbiology Gold Medal of Honour for "most outstanding services to microbiology"—a fitting tribute to a scientist of distinction and a man of great personal achievement.

A man of wide interests and ability he was an accomplished pianist, a keen botanist and, until recently, an enthusiastic and capable cricketer. He spoke French, German and some Norwegian, and read easily in those languages and in Danish. His colleagues will remember him as a man of great integrity, with a mind able to reduce complex questions to simplicity and to answer from a thorough understanding of the basic chemical and physical principles involved. His ability to evaluate arguments logically and to summarise complex discussions concisely made him especially valuable as a chairman at committees.

T. A. Roberts

William Bullerwell

DR WILLIAM BULLERWELL, FRS, FRSE, Deputy Director of the Institute of Geological Sciences, and Chief Geophysicist 1962-1977, died on 25 November aged 61.

After graduating with first class honours in physics in 1937 from Durham University (King's College) he took a further degree in geology, gaining the Lebour Prize for Field Geology, and began research in mining geophysics. This was interrupted by war service during which he

worked on experimental radar, and was mentioned in dispatches.

In the post-war period he joined the Geological Survey of Great Britain, now within the Natural Environment Research Council as part of the Institute of Geological Sciences. Following the pioneer geophysical work for the Survey by J. Phemister and W. F. P. McLintock, he initiated the programme of geophysics that is now a major part of the Institute's work.

In 1950 the Geophysics Division became responsible for collating all gravity data obtained in Great Britain and for its final publication as maps of Bouguer Anomaly: eight quarter-inch to one mile gravity overlay maps were published between 1954 and 1968. During the decade 1955-1965 he supervised the complete regional aeromagnetic survey of the United Kingdom which was published (following interim 1:250,000 scale Diagram Edition sheets) as colour-layered 'ten-mile' aeromagnetic sheets in 1965 and 1972. With his wife, Eileen, whom he married in 1942, and Dr and Mrs James Phemister, during summer vacations from 1953-1956 he carried out the first reconnaissance gravity survey of Scotland.

With the coming of the hydrocarbon search he became deeply involved in the government's research programme of hydrocarbon assessment and search on the continental shelf, especially in advising the Department of Energy. Recently he served on the Advisory Committee of Programme Management for Geothermal Energy of the Commission of European Communities, and from 1970 onwards he was much concerned with offshore prospecting in East Asia as representative for the Ministry of Overseas Development at the Economic and Social Commission for Asia and the Pacific Co-ordinating Committee.

Dr Bullerwell was an exceptionally kindly and generous man who had an encyclopaedic geophysical knowledge that was widely consulted and recognised, and he was elected FRS in 1972 (Council 1974-5) and FRSE in 1973. He served on numerous governmental, university and other professional committees, acting as Treasurer of the Geological Society from 1963-71, and as Chairman of the University of London's Advisory Board on Geophysics.

P. A. Sabine

announcements

Appointments

Lord Todd, O.M., President of the Royal Society has appointed the following Vice Presidents for the year ending 30 November 1977: **Dr B. J. Mason, C.B.**; **Sir Harrie Massey, Professor D. C. Phillips**; **Dr M. G. P. Stoker, C.B.E.**; **Professor P. Allen**; **Dr G. D. H. Bell, C.B.E.**; **Sir Angus Paton, C.M.G.**; and **Sir Peter Swinnerton-Dyer, Bt.**

J. L. van der Post, Director of the British Gas Engineering Station at Killingworth succeeds **Dr R. G. Allen, O.B.E.**, as Director of the Water Research Centre from February.

The following have been elected Emeritus Professors of the University of Bristol: **T. K. Ewer** in Animal Husbandry, **W. A. Gillespie** in Clinical Bacteriology and **R. F. E. W. Peel** in Geography.

Dr Fred Stratton, Director of the Blood Transfusion Service of the North Western Regional Health Authority to Professor (part-time) of human serology at Manchester University.

Awards

The 1977 Papers and Craftsmanship Premiums of the Gordon Radley Fund (Christopher Columbus Prize) awarded by the Post Office to scientists and engineers under 30 for published research work went to **Michel Eve**, **Dr Graham Davies**, **John Davies** and **Robert Walters**.

Michael Heidelberger of New York University, **Elvin A. Kabat** of Columbia and **Henry G. Kunkel** of Rockefeller University have shared the Columbia University's 1977 Louisa Gross Horwitz Prize for their research on the body's disease combating ability and its use in the diagnosis and treatment of disease.

The Paul Martini Prize (20,000 DM) for 1978 will be awarded by the Medizinisch Pharmazeutische Studiengesellschaft e.V., Humboldtstrasse 94, D 6000 Frankfurt. The award is intended to promote the development of scientific methods for the evaluation of clinical, pharmacological and therapeutic fields. Papers, which must be self contained and if published less than 2-yr-old, should be submitted before 30 April 1978.

On the move

Dr Martin Evans from the Department of Anatomy and Embryology, University College London to the Department of Genetics, University of Cambridge.

Dr Norman Shaw from Microbiological Chemistry Research Laboratory, University of Newcastle upon Tyne, to visiting professor in the Department of Chemistry, Washington University.

Dr John Walker from Physics Department, University of Reading and Groupe de Physique des Solides de l'Ecole Normale Supérieure, Université Paris VII, to Quantum Science Corporation, 16 Charles II St, London.

Meetings

3-7 April, **Post-Graduate School on Aspects of Drug Toxicity**, London (R. E. Marshall, Department of Pharmaceutical Sciences, The Pharmaceutical Society of Great Britain, 1 Lambeth High Street, London SE1, UK).

10-14 April, **Symposium on HPLC with application to the Pharmaceutical and Food Industries**, Sunderland (Dr D. Dennis, School of Pharmacy, Sunderland Polytechnic, Chester Road, Tyne and Wear, UK).

12-26 April, **Chromatin Structure and Function**, Erice, Sicily (C. Nicolini, Professor of Biophysics, Temple University, 3223 North Broad Street, Philadelphia, Pennsylvania 19140).

17-20 April, **Institute of Environmental Sciences 24th Annual Technical Meeting**, Texas (B. L. Peterson, Institute of Environmental Sciences, 940 East Northwest Highway, Mount Prospect, Illinois 60056).

19-21 April, **The Origin of Major Invertebrate Groups**, Hull (Dr M. D. Brasier, Department of Geology, The University Hull, UK).

20-26 April, **Remote Sensing of the Environment**, Manila (U-M Extension Service, Conferences and Institutes, 412 Maynard Street, Ann Arbor, Michigan 48109).

25 April, **Fundamental Studies in Keratinisation**, Amsterdam (Professor M. W. Greaves, Institute of Dermatology, Homerton Grove, London E9, UK).

25-26 April, **Some Recent Results in X-ray Astronomy**, London (The Executive Secretary, attn PW, The Royal Society, 6 Carlton House Terrace, London SW1, UK).

6-11 May, **80th Annual Meeting of the American Ceramic Society**, Detroit (The American Ceramic Society Inc., 65 Ceramic Drive, Columbus, Ohio 43214).

9 May, **International Symposium on Crop Protection**, Belgium (Facultéit van de Landbouwwetenschappen, Coupure links 533, B-9000 Ghent, België).

10-12 May, **Biotechnology in Energy Production and Conservation**, Tennessee (Charles D. Scott, Oak Ridge National Laboratory, P.O. Box X, Oak Ridge, Tennessee 37830).

15-17 May, **9th International Symposium on Chromatography and Electrophoresis**, Lake Garda (Dr Alberto Frigerio, Istituto di Ricerche Farmacologiche "Mario Negri", Via Eritrea 62, 20157 Milan, Italy).

15-19 May, **3rd International Conference on Basement Tectonics**, Colorado (Dr Donald L. Baars, Department of Geology, Fort Lewis College, Durango, Colorado 81301).

15-19 May, **1st Australasian Mathematical Convention**, Christchurch (The Convention Secretary, Mathematics Department, University of Canterbury, Christchurch, New Zealand).

Reports and Publications UK & Ireland—November

Building Britain's Future: Labour's Policy on Construction. Pp. 64. 35p. The EEC and Britain: a Socialist Perspective. Pp. 75. 60p. International Big Business: Labour's Policy on the Multinationals. Pp. 135. 90p. (London: Literature Sales, The Labour Party, Transport House, Smith Square, SW1, 1977.)

Progress in Physical Geography, Vol. 1, No. 1, 1977. Pp. 1-184. Subscription rates: UK, Europe and rest of the world (excl. USA and Canada) £12.50; \$25. USA and Canada \$25. (London: Edward Arnold (Publishers) Ltd., 1977.)

The Edinburgh School of Agriculture. Annual Report, 1976. Pp. 203. (Edinburgh: The Edinburgh School of Agriculture, 1977.)

Occasional Reports of the Royal Observatory, Edinburgh. No. 2: Topics in Extragalactic Astronomy with special reference to the Southern Hemisphere. By Gérard de Vaucouleurs. Pp. vi + 125. (Edinburgh: The Royal Observatory, 1977.)

Physical Impairment: Social Handicap. Pp. 32. (London: Office of Health Economics, 162 Regent Street, W1, 1977.)

Department of Health and Social Security and the Welsh Office. Advisory Committee on Alcoholism: Report on Prevention. Pp. 14. (London: Department of Health and Social Security, 1977.)

Science Research Council. The Support of Research and Postgraduate Training in Polytechnics—First Report by the Polytechnics Committee to the Science Research Council. Pp. vii + 51. (London: Science Research Council, State House, High Holborn, WC1, 1977.)

Health and Safety Executive. Health and Safety: Mines and Quarries. Pp. v + 58. (London: Health and Safety Executive, 1977. Available from HMSO.)

Philosophical Transactions of the Royal Society of London. B: Biological Sciences. Vol. 281, No. 978: Anatomical, Physiological and Biochemical Studies of the Cerebellum from reeler Mutant Mouse. By J. Mariani, F. Crepel, K. Mikoshiba, J. P. Changeux and C. Sotelo. Pp. 1-26 + plates 1-16. UK £5; Overseas £5.15. Vol. 281, No. 979: The Microstructure of the Dentition and Dermal Ornament of Three Dipnoans from the Devonian of Western Australia—a Contribution Towards Dipnoan Interrelations and Mesophogenesis, Growth and Adaptation of the Skeletal Tissues.

- By Moya M. Smith. Pp. 29-72 + plates 1-10. UK £4.90; Overseas £5.05. (London: The Royal Society, 1977.) [711]
- The Safety of the Herbicides 2,4-D and 2,4,5-T. By D. J. Turner. (Forestry Commission Bulletin No. 57.) Pp. 36. (London: HMSO, 1977.) £1.20 net. [711]
- Proctor and Gamble Limited. Annual Report for the year ended 30th June 1977. Pp. 9. Some Basic Beliefs About Marketing. A talk by Edward G. Harness. Pp. 21. (Gosforth, Newcastle upon Tyne: Procter and Gamble Limited, 1977.) [811]
- Freshwater Biological Association. A Key to the British Fresh- and Brackish-water Gastropods, with Notes on Their Ecology. By Dr. T. T. Macan. Pp. 46. (Ambleside, Cumbria: Freshwater Biological Association, The Ferry House, Freshwater, 1977.) 60p. [911]
- British Nutrition Foundation Annual Report, 1976/1977. Pp. 26. (London: British Nutrition Foundation, 15 Belgrave Square, SW1, 1977.) [911]
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